University of Alberta

The Effects of Diet and Tracer Administration Route on Tryptophan Requirements of Neonatal Piglets

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Nutrition and Metabolism

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ABSTRACT

Previous research has demonstrated that enterally fed piglets require ~30% more methionine and ~55% more threonine than parenterally fed piglets. To determine whether tryptophan requirements of the piglet is affected by the route of diet infusion, we used the indicator amino acid oxidation (IAAO) technique in newborn piglets receiving an elemental diet either enterally or parenterally. Using breakpoint analysis, the mean tryptophan requirement was determined to be 0.13 (±0.02 SE) and 0.15 (±0.02 SE) g/kg/d for enterally and parenterally fed piglets, respectively. Thus, it is unlikely that tryptophan is preferentially utilized by the gut. However, it has been demonstrated that a substantial portion of phenylalanine (commonly used as an indicator amino acid) is utilized on first pass by the splanchnic bed. To determine whether metabolism of tracer during IAAO affects phenylalanine kinetics and requirement estimates, enterally fed piglets received L-[1-^{14}C-phenylalanine either intragastrically (IG) or intravenously (IV) during oxidation studies. Although estimates of phenylalanine kinetics were significantly affected by tracer administration route, phenylalanine oxidation, when expressed as a percentage of dose oxidized, was similar for piglets in both groups. The mean tryptophan requirements were 0.11 (±0.02 SE) g/kg/d and 0.13 (±0.02 SE) g/kg/d for piglets given IG and IV tracers. Therefore, the use of an IG administered isotopic tracer with breath sampling provides a similar estimate of tryptophan requirements in piglets.

DEDICATION

To Mom and Dad, who have always encouraged me to pursue my goals and strive to be my very best. Love you both.

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1.0 INTRODUCTION

Infants born prematurely present a challenge with respect to medical and nutritional care. Due to advances in medical treatment, up to 90% of very low birth weight infants survive (Andrews et al., 1994). However, early nutritional management may have both acute and long-term effects on growth and development.

Total parenteral nutrition (TPN) is frequently administered to low birth weight infants, largely because their metabolic immaturity or the presence of disease precludes enteral feeding. Amino acid solutions currently used are commonly based on amino acid patterns of reference proteins fed enterally. However, parenteral feeding by-passes liver and gut first pass metabolism, and thus these solutions may be inappropriate for the parenterally fed infant. Threonine and methionine requirements in TPN fed piglets have been shown to be approximately 45% and 69% of their respective enteral requirements (Bertolo et al., 1998; Shoveller et al., 2000). This suggests that TPN feeding does not alter amino acid requirements equally, and so the parenteral requirement for each indispensable amino acid (IDAA) must be empirically determined. Unfortunately, there are several constraints to using infants as subjects in amino acid requirement studies. An established neonatal piglet model (Wykes et al., 1993; 1994; Ball et al., 1996) is an appropriate alternative for estimating the amino acid requirements of newborns.

Currently, the most sensitive methods of determining amino acid requirements are oxidation studies, particularly the indicator amino acid oxidation technique (Zello et al., 1995). These studies involve the infusion of isotopically labelled amino acids and the subsequent measurement of label in breath as well as in blood or urine. Currently, there is

concern that the route of isotope administration (either intravenous or oral) may alter kinetic parameters and ultimately affect requirement estimates. Substantial first pass utilization of labelled amino acid tracers by splanchnic tissues have been demonstrated in enterally fed humans and animals receiving oral tracers (Matthews et al., 1999; Stoll et al., 1998; van Goudoever et al., 2000). The use of oral compared to IV tracers may consequently result in lower plasma tracer enrichments and higher plasma fluxes (Sanchez et al., 1995; Krempf et al., 1990; Hoerr et al., 1991). Although the route of isotope infusion has been shown to affect various kinetic parameters, no one to date has empirically determined whether tracer administration route significantly alters the estimate of amino acid requirement.

In recent years, parenteral (House et al., 1997;1998) and enteral requirements for several indispensable amino acids have been elucidated (Bertolo et al., 1998; Shoveller et al., 2000). Determination of tryptophan requirements will contribute to achieving the overall goal of designing the optimal amino acid profile for both TPN and enterally fed neonates. This determination of tryptophan requirements and clarifying the effects of tracer administration route on requirement estimates, will be the focus of this thesis.

2.0 LITERATURE REVIEW

2.1 INTRODUCTION

The following chapter will review several aspects of amino acid nutrition and metabolism, particularly with respect to tryptophan. The nutritional management of low birth weight infants will be discussed, as will the classification of amino acids and methods used to assess amino acid requirements. The rationale for use of the piglet model will also be reviewed. Amino acid requirements for the enterally and parenterally fed piglet will be compared, and current issues surrounding the administration of intragastric versus intravenous isotope during tracer studies will be examined. Additionally, the metabolism and utilization of tryptophan will be outlined. This review will conclude with an evaluation of previous studies which have estimated tryptophan requirements for humans and piglets.

2.2 NUTRITIONAL MANAGEMENT OF LOW BIRTH WEIGHT INFANTS: TPN

Low birth weight (LBW), very low birth weight (VLBW) and extremely low birth weight (ELBW) infants are those born weighing below 2500 g, 1500 g, and 1000 g, respectively. Although they constitute a relatively small proportion of infants born each year, they have a much greater incidence of morbidity and mortality than infants born at term. Frequently, these infants have a reduced absorptive capacity of the gastrointestinal tract, usually related to one of four conditions: short gut syndrome due to anatomic defects or surgical removal of necrotic bowel, chronic severe diarrhea, or a lack of

sufficient villous surface area (Hay, 1986). Due to these factors, low birth weight infants frequently receive total parenteral nutrition (TPN).

TPN involves the intravenous infusion of nutrients to support the growth and maintenance of tissues. It can be given alone, or in conjunction with enteral feeding. TPN solutions contain glucose, free amino acids, emulsified lipid, vitamins, and minerals. The goal of TPN administration is to provide nutrients to support the growth and development of infants without taxing their immature metabolic and biochemical systems (Rassin, 1986). Due to the fact that an insufficient supply of protein or amino acids limits growth, the provision of adequate dietary amino acids in the ideal pattern is important to promote optimal growth in the premature infant (Brunton et al., 2000).

Several commercial amino acid solutions are currently available (see Table 2.1). Some of these solutions are based on high quality enteral proteins. Vamin (Kabi Pharmacia, Stockholm, Sweden) is an amino acid solution modelled after whole egg protein and Vaminolact (Kabi Pharmacia, Stockholm, Sweden) bases its amino acid profile on that of human milk protein. Two additional solutions, Travasol Blend C (Clintec, Deerfield, Illinois) and Aminosyn PF (Abbott Laboratories, Columbus, Ohio) are based on reference proteins fed enterally, but are modified in order to correct abberations resulting in plasma amino acid profiles. Finally, Trophamine (Kendall-McGraw, Irving, California) is a solution which was predicted by a mathematical model to yield plasma amino acid concentrations similar to breast fed term infants (Heird et al., 1988). Although the amino acid profiles of commercially available solutions may differ, the ratio of essential and non-essential amino acids are similar among TPN solutions and human milk (Rassin, 1986).

Table 2.1 Amino Acid Profile of Commercial Parenteral Amino Acid Solutions (Ball et al., 1996)

Amino Acid	Vamin[1]	Aminosyn[2]	Vaminolact[1] (g / 100g of	Aminosyn PF[2] Amino Acids)	Travasol Blend C[3]	Trophamine[4]
Alanine	4.3	12.9	9.7	7.0	20.7	5.4
Arginine	4.7	9.9	6.3	12.3	11.2	12.2
Aspartate	5.9	0	6.3	5.3	0	3.2
Cysteine	2.0	0	1.5	0	0	0.1
Glutamate	12.9	0	10.9	8.2	0	5.0
Glycine	3.0	12.9	3.2	3.9	10.3	3.6
Histidine	3.5	3.0	3.2	3.1	4.8	4.8
Isoleucine	5.6	7.3	5.5	7.6	6.0	8.2
Leucine	7.5	9.5	10.8	11.9	7.3	14.0
Lysine	5.5	7.3	8.6	6.8	5.8	8.2
Methionine	2.7	4.0	2.0	1.8	4.0	3.4
Phenylalanine	7.9	4.7	4.2	4.3	5.6	4.8
Proline	11.6	8.7	8.6	8.1	6.8	6.8
Serine	10.7	4.2	5.8	5.0	5.0	3.8
Threonine	4.3	5.2	5.5	5.1	4.2	4.2
Tryptophan	1.4	1.6	2.2	1.8	1.8	2.0
Tyrosine	0.7	0.9	0.8	0	0.4	2.3[5]
Valine	6.1	8.1	5.5	6.6	5.8	7.8
Taurine	0	0	0.5	0.7	0	0.2

Manufacturer: [1]Kabi, [2]Abbott, [3]Clintec, [4]Kendall-McGaw.
[5]Approximately 75% of tyrosine present as N-acetyltyrosine.

Current TPN solutions need improving for several reasons (Brunton et al., 2000). It is unclear as to what constitutes an ideal amino acid profile for parenterally fed, premature infants. Although breast milk is often considered to be the 'gold standard' diet of healthy term infants, human milk protein nutrition occurs enterally and exists as whole protein. TPN fed infants, in contrast, receive individual amino acids directly into the venous circulation, thereby by-passing liver and splanchnic first-pass metabolism. Bertolo et al (1998) demonstrated that the threonine requirement of the TPN fed piglets was approximately 55% lower than their enterally fed counterparts. In addition, the parenteral methionine requirement of piglets, in the absence of dietary cysteine, was only 69% of the enteral requirement (Shoveller et al., 2000). Therefore, feeding infants intravenous amino acid solutions modelled after enterally fed reference proteins is unsuitable. In order to improve current TPN solutions, experiments directly quantifying amino acid requirements must be conducted.

2.2.1 AMINO ACID NEEDS OF INFANTS

2.2.1.1 Classification of Amino Acids

There are a total of 20 amino acids for which there are tRNAs and which are therefore incorporated into body protein. These amino acids can be grouped on the basis of their dietary indispensability. In both the neonate and the adult, nine amino acids must be provided pre-formed in the diet to support growth and maintenance of body tissues: isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine (Rose

et al, 1948; Rose, 1957) and histidine (Holt and Snyderman, 1965; Kopple and Swendseid, 1975). The dispensable amino acids, alanine, asparagine, aspartate, glutamate and serine, can be made by the neonate when an adequate amount of dietary nitrogen and carbon is provided. In addition, there are conditionally indispensable amino acids. Both cysteine and tyrosine can be endogenously synthesized from the indispensable amino acids methionine and phenylalanine, respectively, in the adult. However, in the low birth weight infant, the conversion of methionine to cysteine may be impaired due to poor activity of hepatic cystathionase (Sturman et al, 1970). In addition, formula fed infants exhibit low plasma tyrosine concentrations, lower weight gains, and poorer rates of nitrogen retention, therefore suggesting that tyrosine is indispensable for the neonate (Snyderman, 1971). Although arginine and proline are not listed as indispensable amino acids for the healthy infant (WHO, 1985), there is evidence that they may be conditionally indispensable. In young piglets, both arginine and proline are required (Ball et al., 1986; Southern and Baker, 1983; Brunton et al., 1999) and low birth weight infants fed parenteral diets supplemented with arginine were less likely to develop hyperammonemia (Heird et al., 1972). Taurine is not required for body protein synthesis, but prolonged deficiency may lead to retinal abnormalities in children (Geggel et al., 1985). Taurine synthesis may be impaired by low levels of cysteine-sulfinic acid decarboxylase (Sturman and Hayes, 1980). Finally, it has been speculated by Jackson et al (1981) that glycine may be conditionally indispensable for protein synthesis and protein accretion in the low birth weight infant (Pencharz et al., 1996).

2.2.1.2 Methods Used To Assess Amino Acid Requirements

Experimentally-derived estimates of requirements for individual amino acids have been obtained by several methods. These include the classical nitrogen balance (N balance) method, plasma amino acid concentrations and 2 types of amino acid oxidation techniques: direct oxidation and indicator amino acid oxidation. All of these approaches are based on the fundamental principle that if any of the 20 amino acids incorporated into body protein are fed in less than adequate amounts, then protein synthesis is limited by the amino acid present at the lowest level relative to its requirement. This amino acid is termed the 'limiting' amino acid.

N balance is an indirect measure of amino acid requirements. It involves the careful measurement of dietary nitrogen intake, and nitrogen output via urine, feces, integumental losses and other miscellaneous losses (Hegsted, 1976). It is based on the premise that if an indispensable amino acid is limiting, N equilibrium cannot be maintained (Leverton et al., 1956). Subjects are fed test diets containing varying levels of an indispensable amino acid of interest, and N retention (N_{in}- N_{out}) is determined over approximately a two week period. Typically, the requirement is estimated by determining the point where the dose- response curve meets the zero balance line, which represents N equilibrium (Manatt and Garcia, 1992).

There are several criticisms of the N balance technique. Although frequently used, there is currently little adequate information regarding the precision and accuracy of these studies in humans (Young, 1986). Also, due to the fact that the calculated N balance is

the result of the subtraction of two much larger numbers (intake and output), any isolated or systemic errors in sample collection or analysis can significantly effect the outcome measure (Wallace, 1959; Beisel, 1979). Unfortunately, it is likely that N intake is overestimated and excretion underestimated. Ultimately, this leads to overly positive N balances and thus low estimated requirements (Calloway and Margen, 1971; Hegsted, 1976). Finally, these studies require prolonged adaptation to test diets containing deficient or excessive amounts of indispensable amino acids, and thus are inappropriate for use in the premature infant and other vulnerable groups.

Plasma amino acid response curves have also been used to evaluate dietary amino acid needs. This method is based on the observation that the dietary concentration of the limiting amino acid begins to rise in plasma only when intake exceeds the requirement (Zimmerman and Scott, 1965; McLaughlan and Illman, 1967; Mitchell et al., 1968; Young et al., 1971). As graded increases of the limiting amino acid is provided in the diet, plasma concentrations of the limiting amino acid typically appears low and constant, *until* the level required for maximum growth is reached. At this point, plasma levels of the limiting amino acid rise rapidly and linearly with increasing amino acid intake (Young et al., 1971).

There are advantages and disadvantages of using this method. Unlike nitrogen balance techniques, plasma amino acid response curves can be determined over short experimental periods, using unrestrained conditions (Lewis, 1992). Thus, this method may be applied to a variety of subjects and physiological states. However, there must be careful control over several experimental conditions: diet formulation, time of feeding,

time of blood sampling, and handling of blood samples (Lewis, 1992; Rassin and Bhatia, 1992). A further concern is the interpretation of amino acid profiles. Studies in preterm and term infants have demonstrated that plasma amino acids are fairly sensitive indicators of protein intake and protein quality (Raiha et al., 1976; Gaull et al., 1977; Janas et al., 1985). But plasma amino acids are also affected by stress, such as burns, cachexia and fasting (Rassin and Bhatia, 1992). The large number of compounds present in plasma and not included in amino acid analysis may additionally contribute to interpretation difficulties. The concentration of peptides, proteins and bound amino acids in plasma are affected by amino acid intake, and are not normally measured. Finally, the concentration of amino acids in plasma may be quite different than intracellular amino acid concentrations in other tissues (Rassin and Bhatia, 1992). Therefore, there are several potential problems which ultimately influence amino acid requirement estimates based on this method.

Amino acid oxidation studies are based on the principle that any amino acids provided in excess of the needs of protein synthesis are preferentially oxidized (Zello et al., 1995). They involve the intravenous or oral infusion of an isotopically labelled amino acid tracer. The tracer used is an indispensable amino acid labelled at its carboxyl carbon (typically with ^{13}C for humans and ^{14}C for animals). The labelled carbon preferably undergoes only 2 major reactions: either incorporation into protein, or irreversible oxidation to CO_2. Therefore, the oxidation of the labelled amino acid can be quantitatively determined by the analysis of labelled CO_2 in breath (Zello et al., 1995).

In direct oxidation studies, a known amount of labelled indispensable amino acid is given in conjunction with graded levels of the same, but non-labelled amino acid. Oxidation of this test amino acid is low and constant until the 'breakpoint' is reached, at which point the oxidation increases incrementally as intake increases. The breakpoint, or point of inflection, is considered to be the dietary requirement for the test amino acid (Brunton et al., 1998).

The indicator amino acid oxidation (IAAO) technique differs from that of direct oxidation. The test amino acid or amino acid of interest is not labelled; rather, an indicator amino acid is used as the tracer. The indicator is an indispensable amino acid which has an oxidative pathway unrelated to that of the test amino acid. Subjects are fed diets containing graded levels of test amino acid and are given a primed, constant infusion of tracer. As the dietary intake of the test amino acid increases from deficient to acceptable levels, the oxidation of the indictor amino acid decreases linearly, corresponding to the increase in protein synthesis, until requirement for the test amino acid is met. At this point, again referred to as the 'breakpoint', oxidation of the indicator and presumably all other indispensable amino acids becomes low and constant (Brunton et al., 1998). The concept of the IAAO method is outlined in Figure 2.1.

Although oxidation techniques are more sensitive methods of determining amino acid requirements compared to N balance, there are limitations to the direct oxidation method. This oxidation method cannot be used to determine requirements of all indispensable amino acids, due to the fact that several of these amino acids are involved in

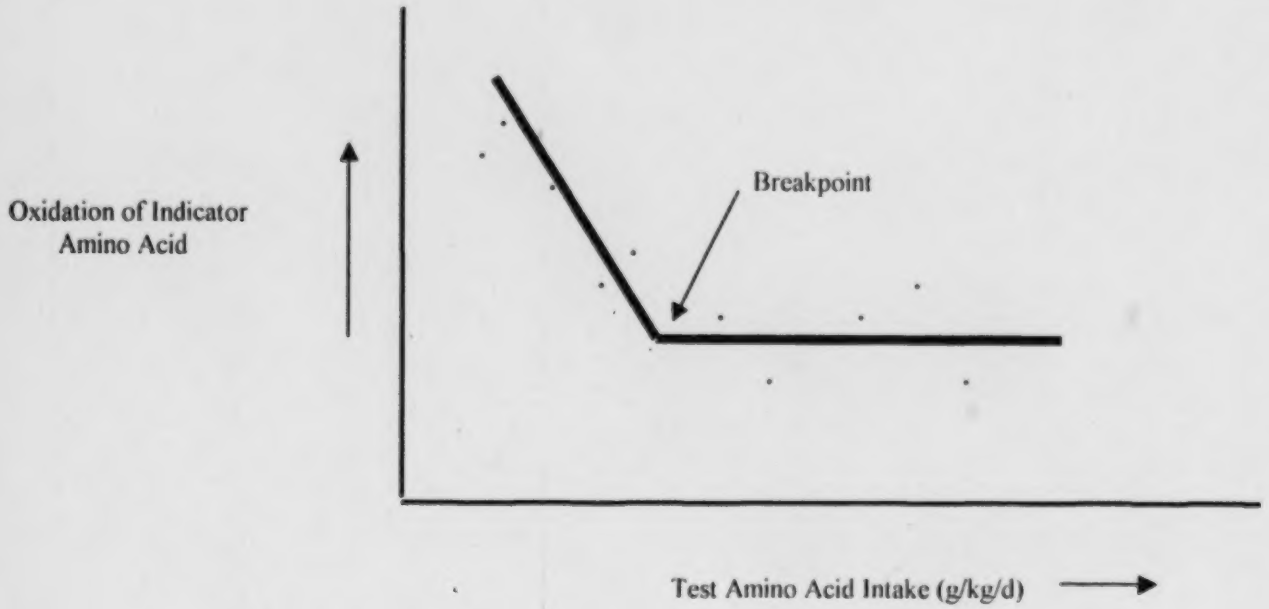

Figure 2.1: Graphic Representation of the IAAO Method

complex metabolic pathways in which the infused label cannot be adequately accounted for quantitatively (eg. methionine, threonine). Additionally, as the dietary test amino acid intake increases, the free amino acid pool size increases as well, thereby leading to dilution of the tracer and an overall reduction in sensitivity (Brunton et al., 1998). Finally, a large dose of ^{13}C- labelled amino acids must be used to compensate for the natural abundance of ^{13}C present in the body (Brunton et al., 1998). Thus, amino acids which are believed to have low requirements, such as tryptophan, cannot be measured by direct oxidation, due to the fact that the amount of isotope used contributes to the dietary intake of test amino acid, and the quantity required for detection may exceed the requirement (Zello et al., 1995).

Fortunately, the IAAO method does not suffer from these criticisms. Oxidation of the indicator amino acid can be easily determined quantitatively, there is no dilution of the tracer as dietary test amino acid intakes increase, and it is possible to test low concentrations of the test amino acid due to the fact the tracer used is the indicator amino acid (Brunton et al., 1998). Lastly, little prior adaptation to test diets are required for this technique. Zello et al (1990) determined that both phenylalanine flux and oxidation was similar for subjects consuming varying levels of phenylalanine over a 6 hour period, after being adapted for 3, 6, or 9 days to two levels of dietary phenylalanine. Such adaptation to differing dietary phenylalanine intakes did not affect the requirement estimate. Overall, the IAAO technique can currently be considered the most sensitive and appropriate technique for determining amino acid requirements, particularly in infants.

2.2.2 THE PIGLET MODEL

There are several ethical and practical constraints to using low birth weight infants as subjects in requirement studies. Due to the immaturity of their metabolic systems, dietary administration of excess or unbalanced amino acids may lead to further metabolic complications, such as hyperammonemia or metabolic acidosis (Rassin, 1986), and thus should be avoided. In addition, the sampling of blood, breath and urine may be restricted, and so oxidation studies may not be clinically feasible (Ball et al., 1996). A further difficulty is that low birth weight infants constitute a heterogeneous population, not only genetically, but also because these infants may suffer from a wide range of illnesses which alter metabolic demands. Understandably, it is difficult to elucidate the effect of small changes in dietary amino acids using such a diverse population (Ball et al., 1996).

The use of a piglet model overcomes the many difficulties of using low birth weight infants as subjects in requirement studies. Healthy piglets are less genetically diverse. The use of piglets enables researchers to explore more comprehensive diet regimens and to use measures that are more invasive, and more sensitive than those used with infants (Ball et al., 1996). Thus, the metabolic effects resulting from potential improvements in dietary amino acid solutions may be further explored using the piglet in place of the infant.

The most important aspect of this model is that piglets and infants are very similar with respect to growth and development. The growth and maturation of the gastrointestinal tract (Moughan and Rowan, 1989; Shulman et al., 1988), the kidney

(Terris, 1986; Glauser, 1966) and the brain (Dobbing and Sands, 1979; Purvis et al., 1982) are similar for both species, as is body composition (Shulman, 1993). Development of the respiratory and haematologic systems are also comparable (Glauser, 1966). According to Moughan and Rowan (1989), premature infants are more similar to neonatal piglets than to full term infants. Indeed, neonatal piglets and premature infants share many physiological characteristics: low birth weight, low fat reserves, low thermoregulatory ability, high metabolic rate (Book and Bustad, 1974), and susceptibility to hypoglycemia (Mount and Ingram, 1971; Ball et al., 1996). The piglet is also an appropriate model with which to study the effects of TPN on the gut (Adeola et al., 1995; Rossi, 1986). TPN feeding has been shown to induce intestinal atrophy, such as decreases in mucosal weight, protein, and villous height (Rossi, 1986).

Protein and amino acid metabolism in piglets and infants are comparable. The concept of metabolic scaling, or extrapolation of whole body metabolism when expressed per unit weight, suggests that the pig is a more appropriate model than smaller animals, such as the rat (Benevenga, 1986). The pattern of amino acids currently estimated for requirements, as well as the profile in milk and tissues, are similar to each other when expressed as a percentage of protein (Ball et al., 1996). The optimal profile of amino acids required by the piglet may be influenced by both protein accretion (growth) and maintenance of tissues on overall whole body protein metabolism. The pattern of amino acids required for growth is similar for neonatal piglets and infants (Ball et al., 1996), due to the fact that the rate of protein synthesis is higher in the young compared to the mature

of each species (Pencharz et al., 1981; Mulvaney et al., 1985; Reeds and Harris, 1981; Benevenga, 1986), reflecting the need for amino acids to support growth over maintenance. Overall, the piglet is an excellent model with which to determine the amount and optimal profile of amino acids needed to promote the healthy development of infants.

Finally, there are several practical benefits to using the piglet model. Piglets are easily obtained from commercial farms for a much lower cost than primates. Unlike primates, these animals can be weaned from their mothers at an early age and they adapt well to artificial rearing environments. Regular care of piglets is relatively cheap, and they are large enough to allow for adequate sampling of biological fluids and tissues (Ball et al., 1996). Thus, the piglet is a more suitable experimental model than the rat or primate.

2.3 AMINO ACID REQUIREMENTS DURING ENTERAL AND PARENTERAL FEEDING

Gastrointestinal tissues substantially influence whole body metabolism. Although the portal-drained viscera constitute 3-6 % of body weight, they are responsible for 20-35 % of whole body protein turnover and energy expenditure (Burrin et al., 1989; Eisemann et al., 1989; Lobley et al., 1980; Stöll et al., 1997; 1999). The maintenance of these tissues is largely dependent on the provision of enteral feeding. When TPN is administered for a prolonged period, gut atrophy is induced (Adeola et al., 1995; Goldstein et al., 1985; Johnson et al., 1975; Shulman, 1988).

As mentioned previously, when nutrients are delivered parenterally, they by-pass

liver and gut first- pass metabolism. Consequently, plasma amino acid concentrations during TPN feeding may be greatly different than when enteral nutrients are provided (Bertolo et al., 1999). There is evidence to suggest that first pass metabolism of the gut may influence whole body amino acid requirements. Bertolo et al. (1998) examined threonine requirements in neonatal piglets fed identical diets either parenterally or enterally. Using the IAAO method, they determined that the threonine requirement for TPN fed piglets was approximately 40 % of the requirement estimated for their enterally fed counterparts. This is supported by Stoll et al. (1998), who measured the appearance of labelled amino acids in piglet portal blood following an intragastric infusion of [U-^{13}C] algal protein in combination with enteral feedings. It was determined that uptake of the gut accounted for 61% of the threonine provided in the oral tracer. Similarly, 35 % of both lysine and phenylalanine were absorbed by the gut on first pass (Stoll et al., 1998). The lysine (House et al., 1998) and phenylalanine (House et al., 1997) requirements of TPN fed piglets has also been determined using the IAAO method. When compared to NRC recommendations for enterally fed piglets, the parenterally fed animals had approximately 30 % lower requirements for both lysine and phenylalanine. Finally, the methionine requirement in parenterally fed piglets was estimated to be approximately 70 % of the enteral requirement based on indicator amino acid oxidation (Shoveller et al., 2000).

Overall, it has been shown that roughly one third of dietary essential amino acids is taken up during intestinal first pass metabolism (Stoll et al., 1998). However, the extent to which each of these indispensable amino acids is absorbed and utilized by the gut differs.

Currently, the proportion of tryptophan initially taken and metabolized by the gut is unknown. Unlike threonine, which greatly contributes to intestinal mucin production (G. Law, MSc. thesis, 2000), tryptophan is not currently believed to be preferentially utilized by the gut. Although dietary tryptophan requirements have been defined for the enterally fed young pig (Firth and Johnson, 1956; Ball and Bayley, 1984) and human (Lazarus-Brunner et al., 1998; Leverton et al., 1956; Young et al., 1971), the parenteral requirement for tryptophan has yet to be elucidated.

2.4 TRACER STUDIES: ORAL VS INTRAVENOUS ISOTOPE

Several isotopically labelled amino acids have been used as tracers to examine amino acid kinetics. Typically, leucine, phenylalanine and lysine are used in oxidation studies, due to the fact that they have reasonably few metabolic fates, and that they have a carboxyl carbon that is irreversibly oxidized, and thus appears in breath CO_2. Amino acid requirement studies, using oxidation techniques, can be conducted with two possible routes of isotope administration: oral or intravenous. IV tracers have been commonly used in pigs (Bertolo et al., 1998; House et al., 1997,1998) as well as in adult humans (Zello et al., 1990,1993; Duncan et al., 1995; Lazarus-Brunner et al., 1998). As stated previously, IV infusion techniques are invasive, and are considered inappropriate for use in special populations, like premature infants. Oral administration of isotope is potentially less invasive. Oral tracers were used in the early development of the IAAO method in pigs (Ball and Bayley, 1984; Kim et al., 1983). Oxidation studies using oral tracers have been

done in adults as well (Basile-Filho et al., 1997; Sanchez et al., 1995; Bross et al., 1998).

There are several issues surrounding the use of oral versus IV tracers. There is substantial first pass metabolism of labelled amino acids by the splanchnic tissues (Matthews et al., 1993; Biolo et al., 1992; Hoerr et al., 1991; Krempf et al., 1990; Stoll et al., 1998; van Goudoever et al., 2000). The fraction of gastrically fed [phenyl-$^{2}H_{5}$]-phenylalanine utilized by the splanchnic bed on first pass was estimated to be approximately 30% in the adult human (Matthews et al., 1999). This measure was derived by examining plasma isotopic enrichment of phenylalanine following the oral administration of tracer.

The gastrointestinal tract is likely the predominant organ involved in splanchnic extraction of amino acids. Using a combination of mass balance and tracer infusion techniques, Stoll et al (1998) determined that approximately 35% of enteral ^{13}C-phenylalanine provided in the diet as [U-^{13}C]-algal protein was taken up on first pass by the gut, with the remainder appearing in the portal blood. This is very similar to previous estimates of entire splanchnic phenylalanine extraction or first-pass removal of amino acids by both the gut and liver (Biolo et al., 1992; Matthews et al., 1999). Indeed, it has been estimated that 75% of total splanchnic metabolism of intragastric phenylalanine tracer is due to gut metabolism in young pigs (Stoll et al., 1997). Similarly, 85% of splanchnic extraction of gastrically administered, labelled leucine is due to gut metabolism, and the remaining 15% due to the liver, in dogs (Yu et al., 1990).

Small intestinal utilization of nutrients, both quantitatively and qualitatively, is

affected by the route of nutrient provision. In the fasting and even the fed state, the portal drained viscera (PDV) use arterial amino acids (Rerat et al., 1992; Yu et al., 1990,1992). Approximately 11, 11, and 5% of the respective intake of labelled lysine, leucine and phenylalanine was taken up by PDV via the arterial circulation in enterally fed piglets (Stoll et al., 1998). What is the metabolic fate of these amino acids absorbed by the gut? Arterially presented labelled amino acid utilization may be different from the use of luminally derived amino acid tracers (Alpers, 1972). As for gastric tracers, only 18, 21, and 18% of ^{13}C-lysine, ^{13}C -leucine and ^{13}C -phenylalanine metabolized by the gut in the first pass was incorporated into mucosal protein (Stoll et al., 1998). For phenylalanine, approximately 50% of the remaining absorbed tracer was likely converted to tyrosine in the mucosa, and the rest may have been oxidized (Stoll et al., 1998). Although the proportion of tracers used for mucosal protein synthesis appears to be relatively similar for different amino acids, the fate of the majority of the tracer amino acid greatly depends upon the metabolic pathways unique to each individual amino acid.

The impact of the gut on tracer metabolism has substantial implications for measurements of amino acid kinetics. The route of isotope infusion may affect the tracer dilution in the plasma pool. There are three possible scenarios which can be considered: the delivery of both diet and tracer intravenously, enteral feeding and intravenous isotope administration, or the use of both diet and tracer intragastrically. Parenteral feeding in combination with IV tracer infusion is considered to be an appropriate means of determining accurate measures of plasma tracer enrichment, flux and oxidation. In this

case, the infusion and sampling of labelled amino acid is in the central or plasma pool. When diet is given enterally and tracer intravenously, the gut preferentially utilizes the unlabelled enteral amino acids on first pass rather than the labelled tracer in the arterial circulation (Stoll et al., 1998). Consequently, plasma enrichment, which can be generalized as the amount of label incorporated into the plasma amino acid pool, may be greater than that of the gut. Considering that the gut may account for over one quarter of whole body protein turnover (Reeds et al., 1999), the lack of tracer utilization by these tissues would likely lead to an underestimation of whole body protein synthesis. Phenylalanine flux is calculated as the dose of labelled phenylalanine divided by the plasma enrichment of the tracer, and therefore disproportionately high plasma enrichments would result in lower rates of phenylalanine flux. Phenylalanine oxidation when calculated using plasma phenylalanine flux, may potentially be lower in enterally fed individuals receiving IV versus IG tracer. Therefore, calculated estimates of whole body phenylalanine oxidation, as well as protein synthesis, may be underestimated when IV instead of IG tracers are used for the enterally fed individual.

Along similar lines, the gastric infusion of diet and tracer may alter amino acid tracer kinetics. As stated previously, approximately 30% of orally administered tracer is taken up on first pass by the splanchnic tissues. As a result, the isotopic enrichment of plasma would be markedly reduced in those receiving IG versus IV tracers. As stated previously, estimations of flux are determined as the dose of tracer divided by plasma enrichment. Consequently, the use of oral tracer compared to IV tracer may result in

lower plasma enrichments and higher plasma fluxes. Several studies have demonstrated significantly lower plasma amino acid fluxes in enterally fed adults receiving IG versus IV tracers (Sanchez et al., 1995; Hoerr et al., 1991; Krempf et al., 1990).

Whether flux is an accurate measure of amino acid kinetics in enterally fed individuals receiving oral tracers is currently unresolved. Is the resulting plasma isotopic enrichment a representation of just the plasma pool or of the whole body pool? Some researchers have corrected flux estimations based on the amount of tracer lost in first pass uptake (Sanchez et al., 1995). In contrast, it may be argued that when both tracer and diet are given enterally, the loss of tracer is a reflection of the amino acids being utilized, and that the subsequent plasma enrichment is more representative of the whole body pool. Clearly, a greater understanding of this area is needed.

The collection of labelled breath CO_2 is a common component of amino acid oxidation studies. When appearance of label in breath is expressed as a percent of dose infused gastrically, the issue of label dilution may be avoided. Perhaps unlike plasma, breath may more accurately reflect intracellular amino acid enrichment. Whether this notion holds true for IV infused tracers given concurrently with enteral diet is unknown. However, recent studies have found no significant differences between whole body oxidation rates of tracers given IV versus IG (El-Khoury et al., 1998; Bross et al., 1998).

The issue of plasma tracer enrichment is critical in direct oxidation studies (J.D. House, PhD. thesis, 1995). However, when the indicator amino acid oxidation method is used, it is the slope of the line and breakpoint in breath CO_2 that is most important in the

determination of the test amino acid (Zello et al., 1995). Consequently, the route of tracer infusion may not be an important issue when indicator oxidation techniques are used, however, no data presently exists to support this point of view.

In conclusion, the route of tracer administration may play a major role in the estimation of amino acid kinetics. First pass metabolism of tracers by splanchnic tissues, primarily the intestine, alter the appearance of labelled amino acids in plasma. Subsequent determination of plasma flux is also affected. The interpretation of whether plasma constitutes the whole body amino acid pool is under question. Although the effect of tracer route on amino acid kinetics has been examined, no one to date has actually compared amino acid requirement estimates directly using either IV or IG tracers.

2.5 TRYPTOPHAN UTILIZATION AND METABOLISM

Tryptophan is a neutral amino acid containing an indole and aromatic side group. Tryptophan is the least abundant amino acid in most proteins (Block and Weiss, 1956), and has been estimated to constitute 1 and 1.5 % of total amino acids in typical plant and animal proteins, respectively (Peters, 1991). Tryptophan is an indispensable amino acid for rats (Rose, 1948), infants and children (Holt and Snyderman, 1965), and adults (Rose, 1957). Tryptophan plays an unique role in protein synthesis, and is a precursor for several important molecules in the body, including niacin, NAD^+, $NADP^+$, and serotonin. Oxidation of this amino acid occurs via one of two main pathways: the kynurenine pathway or through the serotonin pathway. Current tryptophan requirement estimates are

based on several studies conducted in human and pigs. These aspects of tryptophan nutrition and metabolism will be discussed in the following sections.

2.5.1 PROTEIN SYNTHESIS

Tryptophan is one of 20 amino acids required for tissue protein synthesis. Young et al. (1983) estimated the average adult male synthesizes approximately 3 g of protein per kg of body weight (or ~225-250 g protein) daily when at nitrogen equilibrium. This is equivalent to 3.5 g of tryptophan per day that is used for protein synthesis, and three times the average daily tryptophan intake of such individuals. Therefore, a tremendous flux of amino acids course through this pathway, making it quantitatively the most significant utilization of tryptophan (Peters, 1991).

Tryptophan has been suggested to play a unique role in the regulation of protein synthesis. The feeding of a solution containing tryptophan alone stimulated ribosome aggregation and protein synthesis in the liver of rats and mice, whereas solutions of other individual amino acids, including isoleucine, methionine, or threonine did not have such an effect (Sidransky et al, 1971). Others have confirmed that dietary tryptophan may induce hepatic protein synthesis (Park et al., 1973; Jorgenson and Majumdar, 1975; Majumdar, 1982; Ponter et al., 1994), as well as increase the ribosomal fraction of porcine muscle (Lin et al., 1988). The proposed mechanism by which tryptophan stimulates hepatic protein synthesis may be by either increasing mRNA synthesis, or by increasing nucleocytoplasmic translocation of mRNA, thereby increasing the supply of message to

areas in the cell where translocation occurs (Sidransky et al., 1984). These effects may be mediated by tryptophan binding sites on cell nuclei (Cosgrove et al., 1992; Sidransky and Verney, 1996; 1997; Sidransky et al., 1992). The amino acids alanine, phenylalanine, tyrosine, and histidine, as well as the hormone 3,5,3'-triiodothyronine (T_3), have been shown to compete with ^{3}H-tryptophan binding to hepatic nuclei in vitro (Sidransky et al., 1992; Sidransky and Verney, 1999). Unlike tryptophan, these molecules did not stimulate hepatic protein synthesis, but the binding of L-alanine and T_3 to tryptophan receptors on hepatic nuclei prevented the stimulatory effect of tryptophan on hepatic protein synthesis (Sidransky et al., 1990, 1992). Therefore, tryptophan appears to be involved in the regulation of protein synthesis in a manner that is separate from its role as a component of protein.

2.5.2 OXIDATIVE PATHWAY VIA KYNURENINE

Quantitatively, the second most important route of tryptophan metabolism, after protein synthesis, is its oxidation via the kynurenine pathway (Figure 2.2). This pathway accounts for approximately 95% or more of daily tryptophan catabolism (Peters, 1991). The first and rate-limiting step is the irreversible conversion of tryptophan to formylkynurenine. This reaction is catalyzed by tryptophan-2,3-dioxygenase, which is a haem-dependent, liver cytosolic enzyme (Badawy, 1984). Both the amount and activity of tryptophan-2,3-dioxygenase is increased by the presence of tryptophan (Peters, 1991), and this enzyme is also induced by corticosteroids, kynurenine, and glucocorticoids (Knox,

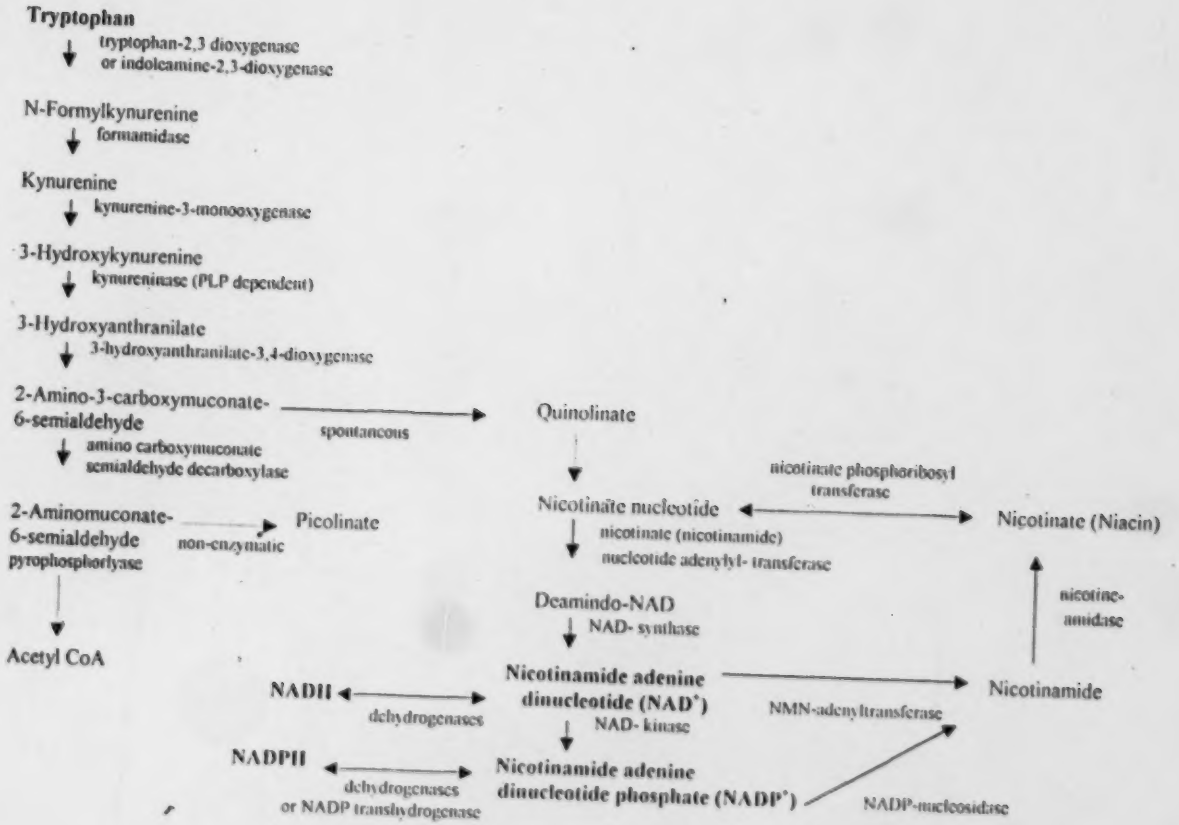

Figure 2.2 The Kynurenine Pathway of Trp Metabolism (adapted from Biochemical Pathways, 3rd edition, Boehringer-Mannheim)

1966; Sainio and Sainio, 1990). Conversely, this reaction is subject to feedback inhibition by NADH and NADPH (Cho-Chung and Pitot, 1967).

A second enzyme is also capable of catalyzing the first step of the kynurenine pathway. Indoleamine-2,3-dioxygenase (IDO) exists in several tissues, including the intestine, stomach, lungs and brain, as well as in macrophages and monocytes (Sainio et al., 1996). This enzyme differs from tryptophan-2,3-dioxygenase because it utilizes superoxide anion instead of molecular oxygen as the oxidizing agent (Hayaishi et al., 1984). IDO has a much broader substrate specificity, and so will act upon not only tryptophan, but also serotonin, tryptamine, and 5-hydroxytryptophan (Hayaishi et al., 1984). IDO is not stimulated by either tryptophan or glucocorticoid (Hayaishi, 1996), but is highly inducible by interferon gamma (IFN-G) (Yoshida et al., 1979, 1981; Ozaki et al., 1987; Takikawa et al., 1990; Werner et al., 1989; Taylor and Feng, 1991).

The extent to which tryptophan is catabolized by IDO varies between species, but in humans, it is believed that tryptophan-2,3-dioxygenase is normally the primary enzyme catabolizing tryptophan (Leklem, 1971; Knowles et al., 1989). However, when the immune system is stimulated, induction of IDO by IFN-G may cause this enzyme to be quantitatively more important in the pathway of tryptophan metabolism. Intraperitoneal administration of bacterial endotoxin induced approximately a 100-fold increase in IDO activity in the mouse lung, and decreased hepatic tryptophan-2,3-dioxygenase to less than 50% of its normal activity (Hayaishi et al., 1984). Induction of IDO in cancer patients receiving IFN-G has been shown to reduce plasma tryptophan concentrations by 50-80%,

although other plasma amino acids remained unchanged (Brown et al., 1989; Byrne et al., 1986; Carlin et al., 1989). Correspondingly, the concentrations of kynurenine and other metabolites of this pathway increase substantially in plasma and urine (5-500-fold increase) (Brown, 1996; Brown et al., 1991). It appears that although the provision of excess dietary tryptophan to the immune challenged may improve plasma tryptophan levels and prevent tryptophan deficiency, supplementation may also further increase the accumulation of kynurenine pathway metabolites. Build-up of these metabolites may result in subsequent complications, such as dementia seen in HIV patients (Brown, 1996).

The next step in the kynurenine pathway is the conversion of formylkynurenine to aminocarboxymuconate-semialdehyde (or acroleylaminofumarate). At this point, the pathway branches. The carbon skeleton of aminocarboxymuconate-semialdehyde can be converted to acetyl-CoA and oxidized completely to CO_2 via the Citric Acid Cycle. Alternatively, aminocarboxymuconate-semialdehyde can be converted to quinolate by way of non-enzymatic cyclization (Peters, 1991). Normally, the majority of aminocarboxymuconate-semialdehyde is oxidized to CO_2, and the significant conversion of tryptophan through quinolate to niacin only occurs when the capacity of one of the enzymes in the former pathway (namely, aminocarboxymuconate-semialdehyde decarboxylase) becomes limiting (Bender, 1982).

There are several important compounds that are produced via the kynurenine pathway, each of which have specific functions in the body. Picolinate is produced by the non-enzymatic cyclization of aminomuconic semialdehyde, and it plays a role in intestinal

zinc absorption (Evans and Johnson, 1980). Quinolinate is an iron chelator, and is involved in the regulation of gluconeogenesis (Veneziale et al., 1967). Niacin (vitamin B_3), otherwise known as nicotinate, is produced by the further metabolism of quinolinate (Figure 2.2). If dietary niacin intake is insufficient, 60 mg of tryptophan is required for each 1 mg of niacin formed (Horwitt et al., 1956).

Niacin's coenzyme derivatives, Nicotinic Adenine Dinucleotide (NAD^+) and $NADP^+$, are other products of tryptophan metabolism. Both of these coenzymes are ubiquitous, yet they have different functions. NADH transfers electrons from intermediate molecules into the electron transport chain, and NAD^+ participates in several oxidative reductions, including: glycolysis, oxidative carboxylation of pyruvate, oxidation of acetate through the Citric Acid Cycle, and the beta-oxidation of fatty acids. NADPH is involved in the reductive biosynthesis of fatty acids, cholesterol, steroid hormones, glutamate and deoxyribonucleotides (Hunt and Groff, 1990). A further metabolite of NAD^+ is poly(ADP-ribose). Poly(ADP-ribose) is a homopolymer of ADP-ribose which is necessary for the repair of damaged DNA (Hayaishi et al., 1984). Therefore, several metabolites produced from the catabolism of tryptophan via the kynurenine pathway, particularly niacin, NAD^+, and $NADP^+$, are of great importance for several daily metabolic processes.

2.5.3 SEROTONIN (5-HT) PATHWAY

The biosynthesis of serotonin occurs in several tissues, including enterochromaffin

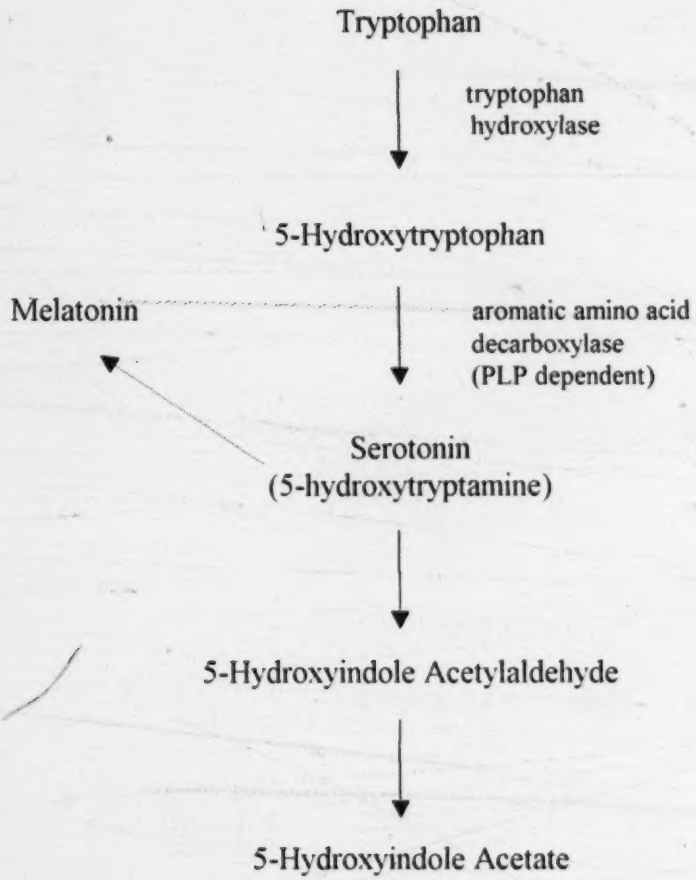

Figure 2.3 Metabolism of Tryptophan to Serotonin
(adapted from Voet & Voet, p. 759, 1995)

cells of the gut, blood platelets and the central nervous system (Peters, 1991). Serotonin synthesis from tryptophan occurs via a two- step process in the brain (Figure 2.3). Under normal conditions, the synthesis of 5-HT is controlled by tryptophan availability within brain cells and, in turn, tryptophan availability is determined by the transport of circulating amino acids across the blood brain barrier (BBB) (Pardridge, 1998). Less than 1 % of dietary tryptophan is utilized for serotonin (5-hydroxytryptamine, 5-HT) synthesis (Peters, 1991). Serotonin is a neurotransmitter and may act as a trophic factor in the developing brain (Emerit et al., 1992), may modulate neural information processing (Soubrie, 1986; Spoont, 1992), and may be involved in the regulation of pain perception, aggressive behaviour, sleep and appetite (Sved, 1983).

An additional metabolite, tryptamine, is a trace amine formed from the direct decarboxylation of tryptophan (Sainio et al., 1996). Brain concentrations of tryptamine are lower than those of serotonin; however, tryptamine influences the effect of serotonin on neurons (Boulton, 1979). Serotonin can be further metabolized into melatonin in the pineal gland. Melatonin plays a role in reproductive and immune functions (Reiter, 1991; Maestroni, 1993), food intake (Ayles et al., 1996; Bubenik and Pang, 1994) and digestive functions (Bubenik, 1986; Bubenik and Dhanvanatri, 1989).

2.5.4 TRYPTOPHAN ABSORPTION AND TRANSPORT

Tryptophan is classified as a large neutral amino acid (LNAA). In the epithelial cell membranes of the intestinal and renal brush borders, tryptophan is transported from

the lumen to the enterocyte via System B. System B is a broad specificity system which uses sodium to transport branch chain (BCAA), aromatic and aliphatic amino acids (McGiven, 1996). Tryptophan is also transported by the sodium independent System L, found in several tissues throughout the body. System L transports primarily BCAA and aromatic amino acids (including tryptophan, phenylalanine, and tyrosine) (McGiven, 1996).

In muscle, the K_m of LNAA transport systems is typically greater than plasma neutral amino acid concentrations. Consequently, substrate competition does not occur in this tissue (Peters, 1991). The uptake of tryptophan into liver cells, as well, is not sensitive to competitive inhibition by other LNAA. However, such competition occurs in the brain. This is due to the fact that plasma tryptophan concentrations are similar relative to the K_m of LNAA transport carriers of the blood brain barrier. Therefore, both the absorption and utilization of dietary tryptophan may be affected by the presence of other large neutral amino acids.

Unlike other amino acids, approximately 80-90% of plasma tryptophan is bound to albumin (McMenamy and Oncley, 1958). Free fatty acids (McMenamy, 1964), bilirubin (McArthur et al., 1971), and various drugs (Lewander and Sjostrom, 1973; Spano et al., 1974; Iwata et al., 1975; Muller and Wollert, 1975) can bind to tryptophan binding sites on albumin and increase plasma free tryptophan levels. Normally, only free plasma tryptophan is used for metabolism in the liver (Smith and Pogson, 1980) and striated muscle (Lehnert and Beyer, 1991). In contrast, most albumin bound tryptophan can be

transported across the blood brain barrier (Pardridge, 1998). Although the survival advantage of albumin binding is unclear, it has been speculated that when plasma tryptophan concentrations are low, tryptophan binding to albumin may prevent hepatic catabolism of this amino acid, thereby allowing for an adequate supply to peripheral tissues, particularly the brain (Peters, 1991).

2.5.5 TRYPTOPHAN REQUIREMENT STUDIES

For over the last 50 years, researchers have tried to determine the requirements of individual indispensable amino acids for humans, as well as for animals, including the pig. Tryptophan requirement studies have been conducted in the infant, child, and both the male and female adult (Holt and Snyderman, 1965; Rose, 1957; Leverton et al., 1956; Lazarus-Brunner et al., 1998). In the pig, several studies have been conducted in order to determine ideal tryptophan intakes for a variety of size and age groups. The National Research Council has extensively compiled these studies in their current report on the Nutrient Requirement of Swine (1998). Requirement studies for both humans and pigs will be discussed, with special emphasis on the tryptophan needs of infants and neonatal piglets.

2.5.5.1 Tryptophan Requirements of Humans

Few studies have examined tryptophan requirements in infants and children. The earliest study was conducted by Albanese and coworkers (1947), on three male infants

who ranged in age between 6 and 12 months. They were fed a hydrolyzed casein diet, supplemented with cystine, and graded amounts of tryptophan were added. It was found that dietary levels of 6 mg tryptophan / kg body weight was insufficient to support normal growth and nitrogen retention, and that 59 mg/ kg was greater than adequate. One of the infants studied appeared to have a requirement between 23 and 40 mg tryptophan/ kg. It was concluded that the infant requires approximately 30 mg tryptophan/ kg daily for normal growth.

Snyderman and colleagues (1961) studied five male and two female infants using the nitrogen balance technique. The infants were fed a synthetic diet containing 18 amino acids in a pattern which was similar to that of breast milk. The tryptophan content of the diets were decreased gradually, and the nitrogen lost through tryptophan removal was replaced by the addition of glycine. At intakes of 22 mg tryptophan/ kg, all of the infants maintained normal weight gain and nitrogen retention, and at 16 mg/ kg, approximately half of the infants continued to grow well and retain nitrogen. At 13 mg/ kg, however, three of the infants showed poor nitrogen balance. Therefore, Snyderman and coworkers suggested that the normally growing infant likely requires 22 mg tryptophan/ kg daily (Snyderman et al., 1961; Holt and Snyderman, 1965).

In term infants, the tryptophan requirement for growth may range from 13-40 mg/ kg/ d. The latest report from the FAO/WHO/UNU (1985) committee recommends that infants from 0-1 year of age need 17 mg tryptophan/ kg daily for net deposition of new protein into tissues and to balance any losses of nitrogen and amino acids. This report,

however, makes no provisions for the dietary treatment of low birth weight infants, likely because no requirement studies have been conducted on this population. Consequently, estimates of amino acid requirements for low birth weight infants are based on extrapolations from the requirements of normal term infants. The tryptophan requirement of the low birth weight infant has been estimated to be 64, 36, or 49 mg/ kg/ day when infants are fed human milk, bovine milk or modified bovine milk containing 2.8 g protein/ kg of body weight/ day (Heird and Kashyup, 1998). The differences in these estimates of tryptophan requirements is likely due to the quality of protein fed. From these estimates, it appears that the tryptophan requirement of low birth weight infants may be greater than that of normal term infants, a reflection of the premature infant's greater rate of protein synthesis and turnover.

Additional work has been done examining the tryptophan requirements of children and adults. These estimates are summarized in Table 2.2. Clearly, the majority of estimates have been derived from nitrogen balance studies. Consequently, the current FAO/WHO/UNU (1985) tryptophan recommendations for the adult is set at 3.5 mg/kg/d. It has been argued that these requirement estimates may be too low, for nitrogen balance techniques likely underestimate true amino acid requirements by a factor of 2-3 times (Hegsted, 1976; Young, 1986). Indeed, Lazarus-Brunner and colleagues (1998) determined the tryptophan requirement of adult females to be 4.0 mg/kg/d using the indicator amino acid oxidation method. This estimate is 79 % higher than those determined using nitrogen balance.

Table 2.2 Tryptophan Requirement Estimates in Humans

Age	TRP Requirement (mg/kg/d)	Technique Used	Source
< 1 yr	30	N-balance	Albanese et al., 1947
< 1 yr	22	N-balance	Snyderman et al., 1961
10-12 yrs	3.7	N-balance	Nakagawa et al., 1963
Adult (female)	2.8	N-balance	Leverton et al., 1956
Adult (female)	4.0	Indicator oxidation	Lazarus-Brunner et al., 1998
Adult (male)	3.6	N-balance	Rose et al., 1954
Adult (male)	3.0	Plasma concentrations	Young et al., 1971
Elderly	2.0	Plasma concentrations	Tontisirin et al., 1973

Overall, a great deal of research has been conducted in man to determine amino acid requirements at several ages. The requirement of indispensable amino acids, including tryptophan, appears to be markedly reduced by adulthood when expressed in relation to body weight (Table 2.3). Young and Pellett (1987) have argued that requirements for both adults and 2-5 year old children would be similar when requirements are expressed per unit of total protein requirement.

2.5.5.2 Tryptophan Requirements of Pigs

The metabolic similarities between humans and pigs justify an examination of tryptophan requirements in the young pig. An extensive array of requirement experiments have been conducted in pigs of all sizes, using several feeds and techniques. Tryptophan requirement studies conducted on piglets weighing up to 5 kg are listed in Table 2.4.

Ball and Bayley (1984) measured the tryptophan requirement of 2.5 kg piglets using the indicator amino acid oxidation method. Piglets were fed diets containing a mixture of skim milk and amino acids. Graded levels of tryptophan, ranging from 0.65-3.0 g / kg diet were given prior to the oxidation period. Using a two-phase linear crossover model, the tryptophan requirement was determined to be 2 g/ kg of a 240 g protein per kg diet. This is equivalent to 0.20 % of the diet as fed (NRC, 1988).

The remaining studies on young piglets (1-5 kg) used weight gain and feed efficiency parameters in the determination of tryptophan requirements. Although these studies differ considerably in approach from Ball and Bayley (1984), requirement estimates

Table 2.3 Amino Acid Requirements of Humans According to Age

Age	Tryptophan Requirement (mg/kg/d)	Tryptophan Requirement (mg/g protein)
2-5 yrs	12.5	11
10-12 yrs	3.3	9*
18+ yrs	3.5	5

Source: FAO/WHO/UHU (1985)
* NRC (1974)

Table 2.4 Tryptophan Requirements of Pigs (1-5 kg) *

Weight (kg)	Diet	TRP Requirement (% of diet fed)	Response Criteria	Source
2	semi-purified	0.17	weight gain, feed efficiency	Firth and Johnson, 1956
2.5	semi-purified	0.20	indicator oxidation	Ball and Bayley, 1984
5	corn-fish meal	0.18-0.22	weight gain, feed efficiency	Gallo and Pond, 1966
5	corn-whey-soybean meal	0.18	weight gain, feed efficiency	Zimmerman, 1975
5	complex	0.19-0.23	weight gain, feed efficiency, plasma metabolites	Lewis et al., 1981

* adapted from Nutrient Requirements of Swine (NRC, 1988)

for all of these studies are similar, ranging from 0.17- 0.23 % of the diet as fed. Current NRC recommendations for tryptophan in 1-5 kg piglets are heavily based on these studies, as well as on data extrapolated from larger pigs. The daily tryptophan requirement for a 3 kg neonatal piglet is currently estimated to be 0.153 g/ kg body weight (NRC, 1998).

2.6 SUMMARY

Determining the ideal amino acid profile for premature infants will ultimately promote maximal growth and development without straining their immature metabolic systems. Defining this optimal profile is necessary due to the fact that current amino acid solutions are unsuitable for low birth weight infants who frequently require TPN feeding. TPN administration by-passes liver and intestinal first pass metabolism, and has been shown to result in altered requirements for amino acids when compared to enterally fed animals (Bertolo et al., 1998; Shoveller et al., 2000). Several methods have been used to obtain amino acid requirement estimates. The IAAO technique is a safe and sensitive method of determining requirements (Zello et al., 1995), that overcomes many problems inherent to other measures. Although there are many constraints to using infants as subjects in requirement studies, the neonatal piglet is an appropriate alternative.

The route of tracer administration affects several kinetic parameters in amino acid oxidation studies. This observation has raised concerns regarding the relative appropriateness of oral compared to intravenously infused amino acid tracers. However, a direct evaluation of tracer route on amino acid requirement estimates has not been

completed.

Tryptophan is an IDAA which plays a unique role in the regulation of protein synthesis. It is also a precursor of several important molecules required for normal daily functioning within the body. The tryptophan requirement of the premature infant has not yet been established. Although estimations of enteral tryptophan requirements are available for neonatal piglets, it is currently unknown whether it differs from the tryptophan needs of the parenterally fed neonate.

2.7 HYPOTHESES:

1) There will be no difference in the estimates of tryptophan requirements for piglets fed enterally versus parenterally.
2) There will be no difference in the estimates of tryptophan requirements for enterally fed piglets given IG versus IV tracer.

3.0 DETERMINATION OF TRYPTOPHAN REQUIREMENTS OF THE NEONATAL PIGLET BY INDICATOR AMINO ACID OXIDATION

3.1 INTRODUCTION

Premature birth is a major cause of infant morbidity and mortality. Appropriate nutritional management is an important factor in the care of these infants. However, the biochemical and physiological immaturity of low birth weight (LBW) infants often precludes enteral feeding. Alternatively, total parenteral nutrition (TPN) is used to supply nutrients to this fragile population.

Amino acid solutions currently used in TPN formulas are based on reference proteins fed enterally, such as whole egg protein and human milk protein. Due to the fact that nutrients infused parenterally by-pass both liver and gut first pass metabolism, the use of these amino acid solutions may be inappropriate for parenterally fed infants (Brunton et al., 2000). For example, Bertolo et al. (1998) determined that the parenteral threonine requirement of neonatal piglets was approximately 45% of the mean enteral requirement. In addition, approximately one-third of dietary indispensable amino acids are consumed by the intestine on first pass (Stoll et al., 1998).

Amino acid requirement studies attempt to provide the basis for creating the ideal amino acid profile. The most sensitive method currently employed to define requirements is the indicator amino acid oxidation (IAAO) technique. The use of this method, however, is limited for LBW infants due to several ethical and practical constraints: prolonged

adaptation to a diet deficient or in excess of an amino acid, intravenous tracer infusion, and frequent blood sampling (Ball et al., 1996). Fortunately, the neonatal piglet is an appropriate model for the premature infant, due to similarities in body composition as well as in the growth and development of several organ systems (Wykes et al., 1993).

Our objective was to determine the requirement of tryptophan in the neonatal piglet using the IAAO technique. Tryptophan is an indispensable amino acid that plays an important role in protein synthesis, as well as in the formation of serotonin, niacin, and its coenzyme derivatives NAD^+ and $NADP^+$. Unlike threonine, which is involved in the production of gut mucins, tryptophan has not been implicated to play a major role in gut function apart from protein synthesis. However, it is important to determine if enteral and parenteral requirements differ, so that accurate amino acid profiles can be established for infants fed both enterally and parenterally.

3.2 OBJECTIVES

1) To determine the tryptophan requirement for neonatal piglets fed an enteral, complete diet using the indicator amino acid oxidation technique.
2) To determine the tryptophan requirement for neonatal piglets fed parenterally using the indicator amino acid oxidation technique.

3.3 METHODS

3.3.1 Study Design

Two experiments were conducted for this study. In the first experiment, we determined the tryptophan requirement of piglets fed an enteral, complete diet. In experiment 2, we determined the tryptophan requirement of piglets fed total parenteral nutrition (TPN), which was identical in composition to the enteral diet. The piglets were assigned to dietary tryptophan intakes using a completely randomized crossover design (Figure 3.1).

3.3.2 Animals and Surgical Procedures

The experimental protocols were conducted in accordance with the Canadian Council of Animal Care and approved by the local animal care committee. In experiment 1, male Yorkshire piglets (N=18) were obtained from Shooter's Hill Livestock Inc. (Calmar, AB), and were transported in a heated vehicle to the Metabolic Unit at the University of Alberta. For the second experiment, 18 Yorkshire piglets (7 male, 7 female) were obtained from the University of Alberta's Swine Unit. All piglets were sow fed for 1.5 ± 0.5 days. Piglets, weighing approximately 1.5 kg, were pre-medicated with atropine (0.09 mg/kg intramuscularly (IM)) and anaesthetized with ketamine (22 mg/kg IM) and acepromazine (0.5 mg/kg IM). They were then maintained with 0.8 % halothane. Under

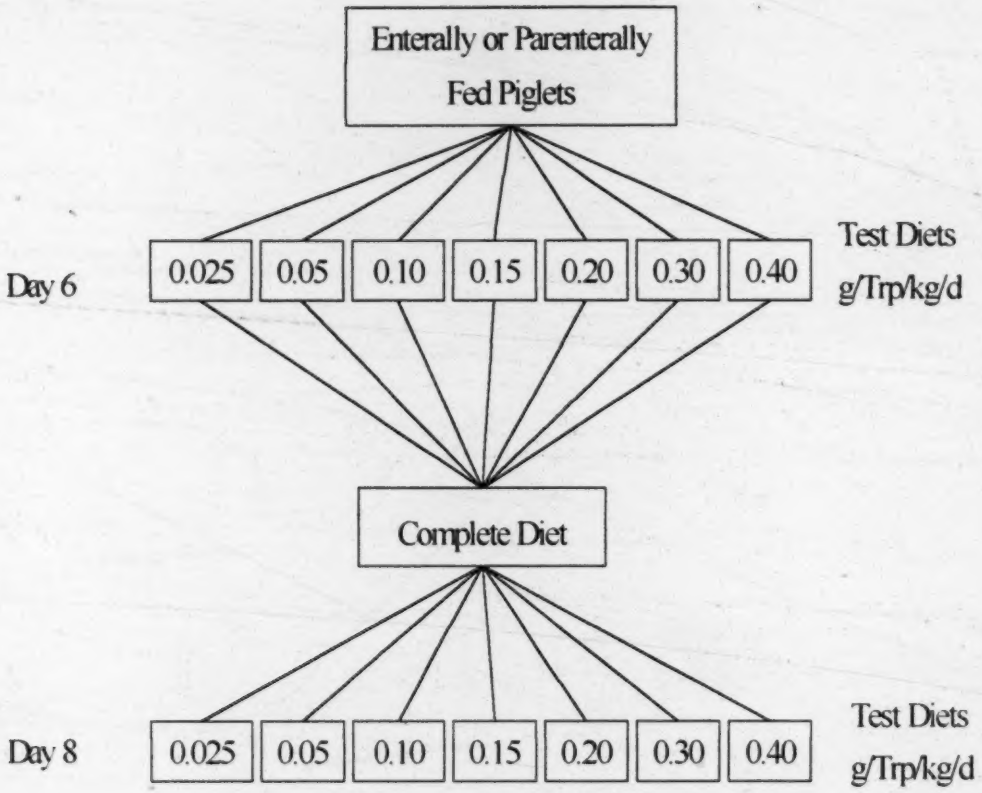

Figure 3.1 Study Design for Experiments 1 & 2: Enteral Tryptophan Requirement Study (IV Tracer) and Parenteral Tryptophan Requirement Study

aseptic conditions, animals underwent catheterization of the jugular and femoral veins. Piglets in experiment 1 also received a gastric catheter. The jugular catheter (1.0 mm i.d. x 2.2 mm o.d.) was used for tracer infusion in experiment 1, and was used for both TPN and tracer infusion in experiment 2. The femoral catheter (0.4 mm i.d. x 1.6 mm o.d.) was used for blood sampling, and the gastric catheter was used for diet feeding in experiment 1. After surgery, each piglet was fitted with a cotton jacket in order to secure and protect the venous lines. Post-surgical antibiotic was administered intramuscularly, and stitches were treated liberally with Hibitane ointment (Ayerst Laboratories, Montreal, P.Q.).

3.3.3 Housing Conditions

Each piglet's cotton jacket contained an anchoring button that was attached to a tether-swivel system secured to the top of the cage. This system prevented the tangling and occlusion of venous lines, while allowing the piglets freedom of movement in the round metabolic cages in which they were housed. The cages were arranged in groups of 4 so that piglets maintained both audio and visual contact with each other. Each cage was equipped with a heat lamp in order to maintain a temperature of approximately 32^0 C, and light was provided between 08:00-22:00. Towels and toys were placed in the cages to provide environmental enrichment to the animals.

3.3.4 Diet Regimen

The elemental diet used was based on that developed by Wykes et al. (1993), with some modifications. The amino acid, vitamin and mineral composition of the elemental diet is listed in Tables 3.1- 3.3. The diet provided 1.1 MJ available energy/ kg/day and 15.6, 27.4, and 9.4 g of amino acids, glucose and fat /kg body weight/day, respectively. All animals received the diet, either enterally or parenterally, immediately following surgery until approximately 21:00 on day 5. At this time, animals were randomly assigned to receive one of 7 test diets containing one of the following levels of tryptophan: 0.025, 0.05, 0.10, 0.15, 0.20, 0.30, or 0.40 g/kg body weight/ day. These levels were chosen due to the fact that the NRC (1998) recommends a 3 kg piglet receive 0.15 g tryptophan /kg/d. This estimate is based heavily on work done by Ball and Bayley (1984), who used the IAAO technique to determine the tryptophan requirement of 2.5 kg piglets consuming a semipurified diet. Therefore, we believed the tryptophan requirement of TPN fed piglets would be similar. In order to ensure all solutions were isonitrogenous, L-alanine was added in place of tryptophan when necessary (Table 3.4). After the oxidation period was completed on day 6, piglets were returned to their cages and infusion of the complete diet was resumed. At approximately 21:00 on day 7, piglets were again randomly assigned to one of the 7 test diets. A second oxidation period was completed on day 8 (see Figure 3.1).

Table 3.1 Amino Acid Content of Complete Elemental Diet

Amino Acid	NRC 1998 † (g/kg/day)	Diet (g/kg/d)	Diet* (g/L)
alanine	----	1.60	5.88
arginine	0.37	1.20	4.41
aspartate	----	0.91	3.36
cysteine	0.26	0.22	0.80
glutamate	----	1.58	5.81
glycine	----	0.71	1.36
histidine	0.29	0.47	1.71
isoleucine	0.49	0.69	2.55
leucine	0.91	1.57	5.76
lysine	0.89	1.25	4.60
methionine	0.24	0.29	1.07
phenylalanine	0.53	0.48	1.76
proline	----	1.25	4.58
serine	----	0.84	3.09
taurine	----	0.07	0.25
threonine	0.53	0.80	2.93
tryptophan	0.15	0.32	1.17
tyrosine	0.31	0.41	0.43
valine	0.59	0.80	2.93
gly-tyr	----	0.39	1.43
Total AA		15.56	54.83

† NRC requirements based on a diet concentration of 4224 kcal/kg (ME) and a daily energy intake of 864 kcal (ME); values based on a 3 kg piglet.

* Composition of diet (g/L) based on intake of 272 mL/kg/d; values based on estimated needs of a 3 kg piglet.

Table 3.2 Vitamin Content of Complete Elemental Diet

Vitamin	NRC 1998 ƒ (mg/d)	Diet ‡‡ (mg/d)	Diet ‡‡ * (mg/L)
Vitamin A (RE)	0.1878	0.4137	0.507
Vitamin D_3 (cholecalciferol)	0.0013	0.0059	0.0073
Vitamin E (DL-α-tocopheryl-acetate)	4.12	4.1283	5.0592
Vitamin K (menadione)	0.11	0.1185	0.1452
Biotin	0.02	0.0118	0.0145
Choline	160	1020	1250
Folacin	0.07	0.0828	0.1015
Niacin	4.81	10.0544	12.3216
Pantothenate	2.82	2.9298	3.5904
Riboflavin	0.95	0.7990	0.9792
Thiamin	0.33	1.0654	1.3056
Vitamin B_6	0.49	0.5993	0.7344
Vitamin B_{12}	0.0046	0.0072	0.0088

‡‡ Vitamin content of diet based on J.D. House (PhD thesis, 1995) for a 3 kg piglet.

ƒ NRC requirements based on a diet concentration of 4224 kcal/kg (ME) and a daily energy intake of 864 kcal (ME) for a 3 kg piglet.

* Composition of diet (mg/L) based on intake of 272 mL/kg/d

Table 3.3 Mineral Content of Complete Elemental Diet

Mineral	NRC 1998 ƒ (mg/kg/d)	Diet * (mg/L)
Calcium	640	500.20
Phosphorus	490	442.20
Sodium	210	714.50
Chlorine	220	1101.9
Magnesium	27	64.63
Potassium	200	1049.2
Chromium	----	0.030
Copper	0.43	2.37
Iodine	0.01	0.054
Manganese	0.33	1.545
Selenium	0.023	0.12
Zinc	7.63	27.80

ƒ Mineral recommendations (NRC) were estimated to meet the upper requirement limit of a 3 kg piglet receiving an enteral diet

* In diet: minerals provided at 200% of that recommended by NRC 1998

Table 3.4 Amino Acid Composition of Test Diets

Amino Acid	Complete Diet (g/kg/d)	Test Diets (g/kg/d) * 1	2	3	4	5	6	7
Alanine	1.60	1.73	1.72	1.70	1.67	1.65	1.61	1.56
Tryptophan	0.32	0.025	0.05	0.10	0.15	0.20	0.30	0.40

Amino Acid	Complete Diet (g/L)	Test Diets (g/L) * 1	2	3	4	5	6	7
Alanine	5.88	6.36	6.32	6.24	6.16	6.08	5.91	5.75
Tryptophan	1.18	0.09	0.18	0.37	0.55	0.74	1.10	1.47

* Test diets contained the identical amino acid profile as complete diet with exception of alanine and tryptophan

3.3.5 Oxidation Periods

Tracer infusion and sample collection during oxidation periods were based on the methods of House et al. (1997), with some minor modifications. On days 6 and 8, animals were transferred to covered plexiglass boxes. After a 30 minute acclimation period, phenylalanine flux and oxidation was determined by a primed (5 μCi/kg), constant (3.5 μCi/kg/h) infusion of an IV tracer solution containing 2.5 μCi/mL of L-[1-^{14}C]-phenylalanine. Air was drawn from the boxes by pump and the total amount of $^{14}CO_2$ expired was trapped in a series of gas washing bottles containing CO_2 absorber (ethanolamine and ethylene glycol monomethylether, 1:2, v/v). Blood samples (1.5 mL) were taken immediately prior to, and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 hours after initiation of label infusion. Blood samples were centrifuged, the plasma was collected and then stored at -20^{0} C until later analysis for phenylalanine specific radioactivity. On day 8, the second oxidation period, blood samples were taken at 60 and 30 minutes prior to label infusion to measure background radioactivity from the infusion on day 6. In addition, background breath samples were collected for 30 minutes at 45 and 15 minutes prior to label infusion. These blood and breath samples were used to correct the results of the second oxidation period for residual radioactivity. Immediately following the oxidation period on day 8, animals were given a lethal dose (750 mg) of sodium pentobarbital through the venous sampling line.

3.3.6 Analytical Procedures

The rate of $^{14}CO_2$ production by animals was determined by liquid scintillation counting of radioactivity in the CO_2 absorbing solution (1 mL absorber:5 mL Atomlight liquid scintillant). Counts were corrected for background radioactivity.

The specific radioactivity of phenylalanine and tyrosine in plasma was determined by reverse-phase high performance liquid chromatography (HPLC), based on the methods of J.D. House (PhD thesis, 1995). In 1.5 mL eppendorf tubes, 300 μL of plasma was combined with 40 μL of an internal standard (2.5 μmol/mL norleucine in 0.1 N HCl), and 1 mL of 0.5% trifluoroacetic acid (TFA) in methanol to prompt protein precipitation. The samples were vortexed, centrifuged at 5000 rpm for 5 minutes, and the supernatants were decanted into 5 mL plastic test tubes. The tubes were then frozen with liquid nitrogen and freeze-dried. In order to determine response factors, 2 equimolar solutions of tyrosine, phenylalanine and the internal standard norleucine were prepared. Once dried, the samples were mixed with 100 μL of an amino acid elution solution containing triethylamine (TEA), methanol, and water in a 1:1:3 ratio. Samples were again centrifuged, frozen in liquid nitrogen, and freeze-dried. The dried samples were mixed with 50 μL of a derivatizing solution containing water, TEA, methanol, and phenylisothiocyanate (PITC) in a 1:1:7:1 ratio. Following a 35 minute derivatization period, these samples were centrifuged, frozen and freeze-dried.

The dried samples were resuspended in 200 μL of sample diluent (5% acetonitrile, 95% phosphate buffer), and were analyzed by reverse-phase HPLC. For each sample, an

100 μL aliquot was injected onto a C^{18} column heated to 46°C. The amino acid derivatives were detected at a wavelength of 254 nm, and both gradient control and peak integration was carried out by a computing software package.

Phenylalanine and tyrosine peaks were collected using an in-line fraction collector. For each peak, three 1 mL fractions were deposited into scintillation vials and mixed with 5 mL of liquid scintillant (BCS). The radioactivity in each vial was determined by counting on a liquid scintillation counter for 10 minutes, using a program for ^{14}C analysis.

Plasma amino acid concentrations were also determined using the method described above, with two modifications: initially, 200 μL of plasma was mixed with the norleucine standard and protein precipitating solution, and 50 μL aliquots of the final derivatized sample were used for HPLC analysis.

3.3.7 Calculations

All calculations were based on the work of House et al. (1997). Amino acid concentrations were determined using the following equation:

[Amino acid](μmol/L) = (Amino acid peak area/ Norleucine peak area)*CF*RF.

In this equation, the concentration factor (CF) is 250 μmol/L and the response factor for the respective amino acid is calculated as:

RF= Norleucine peak area/ Amino acid peak area

for equimolar standards.

The plasma SRA of tyrosine and phenylalanine were calculated using the

following:

SRA (dpm/μmol) = Amino acid radioactivity (dpm/L) / [Amino acid] (μmol/L).

Phenylalanine flux and it's components were determined by the following model of amino acid metabolism:

$$Q = S + E = B + I$$

in which Q, S, E, B, and I denote flux, non-oxidative losses (a reflection of protein synthesis), oxidative losses, contribution from protein breakdown and phenylalanine intake, respectively, at steady state conditions. Phenylalanine flux was determined by:

Q (μmol/kg/h) = Dose (dpm/kg/h) / Plateau (dpm/μmol)

where dose represented the total radioactivity infused.

The $^{14}CO_2$ expiry rate (dpm/kg/h) was determined and the retention of label in bicarbonate pool was corrected using a bicarbonate retention factor (BRF) of 0.93 determined by Wykes et al. (1993). The resulting equation appears as follows:

Corrected $V^{14}CO_2$/kg/h (dpm/kg/h) = $V^{14}CO_2$ (dpm/kg/h) / BRF.

The remainder of the flux components were calculated as follows:

E (μmol/kg/h) = Corrected $V^{14}CO_2$/kg/h (dpm/kg/h) / Dose (dpm/kg/h) * Q

Fraction oxidized (%) = E / Q * 100

S (μmol/kg/h) = Q - E

I (μmol/kg/h) = [Phe] in diet (μmol/mL) * diet infusion (day 6 or 8) (mL/kg/h)

B (μmol/kg/h) = Q - I

3.3.8 Statistical Analysis

Both experiments had a completely randomized design, with the tryptophan level in the diet acting as the main treatment effect. The requirements were estimated using breakpoint analysis with a two-phase linear regression crossover model, and the level of safe intake was determined with 95% confidence intervals (see Appendix). Differences among dietary treatments with respect to gender, day of oxidation, initial weight, final weight, and average daily gain were examined using analysis of variance (ANOVA). Tukey's multiple comparison tests were used to compare plasma amino acid concentrations and estimates of amino acid kinetics between dietary treatment groups. Finally, ANOVAs were used to compare all of these parameters between enterally and parenterally fed piglets.

3.4 RESULTS

3.4.1 Weight Changes

Piglets were active and healthy throughout each of the experiments. Average initial weight at time of surgery was 1.55 and 1.73 kg (SD:± 0.16 and ± 0.15 kg) respectively, for enterally and parenterally fed piglets. Average initial weights were not different among dietary treatment groups in each of the experiments, however, the parenterally fed piglets had a significantly greater initial weight than gastrically fed piglets ($p<0.05$). The mean final weights (on day 8) for enterally and parenterally fed piglets were 2.74 kg and 2.86 kg (SD:± 0.29 and ± 0.26 kg), respectively. The average daily gain for piglets given enterally administered diet was 0.17 kg (SD:± 0.03 kg), and was 0.16 kg (SD:± 0.04 kg) for parenterally fed animals. Mean final weight and average daily gain were similar for piglets fed enterally and parenterally ($p>0.05$), and was not significantly different among dietary treatment groups in the enterally fed piglets. In addition, none of the body weight parameters measured, day of oxidation, or gender significantly affected phenylalanine oxidation (when expressed as % of dose oxidized).

3.4.2 Plasma Amino Acids

3.4.2.1 Enterally Fed Piglets

Several plasma amino acid concentrations were significantly influenced by graded tryptophan intakes in enterally fed piglets (Table 3.5). Phenylalanine concentrations

Table 3.5 Plasma Amino Acid Concentrations of Enterally Fed Piglets (IV Tracer)

	Tryptophan Intake (g/kg/d)								
Amino Acid (μmol/L)	0.025	0.05	0.10	0.15	0.20	0.30	0.40	SE	ANOVA p value
Aspartate	25	17	19	18	15	28	12	1.9	NS
Glutamate	207	140	208	154	166	288	128	16.5	NS
Hydroxyproline	65^{cd}	64^{d}	92^{b}	119^{a}	95^{ab}	91^{abc}	99^{ab}	4.3	.0011
Serine	306	235	263	223	193	277	201	17.7	NS
Asparagine	51	41	20	18	17	19	16	3.6	NS
Glycine	979	746	1098	1159	745	985	762	84.0	NS
Glutamine	549^{a}	447^{ab}	213^{b}	177^{b}	161^{b}	223^{b}	172^{b}	35.2	.0177
Taurine	277^{a}	204^{ab}	148^{bc}	135^{bc}	100^{c}	136^{bc}	136^{bc}	12.5	.0123
Histidine	87	51	43	30	42	62	39	5.0	NS
Citrulline	119^{a}	104^{ab}	85^{abc}	89^{abc}	57^{c}	64^{bc}	71^{bc}	6.1	.0131
Threonine	495	439	817	830	856	872	883	62.3	NS
Alanine	583^{bc}	431^{c}	655^{bc}	758^{abc}	800^{ab}	1022^{a}	784^{ab}	44.8	.0012
Arginine	149	107	158	149	135	95	141	9.1	NS
Proline	739	480	546	537	474	717	583	35.0	NS
Tyrosine	135^{a}	122^{a}	48^{b}	22^{b}	21^{b}	24^{b}	18^{b}	9.7	.0006
Valine	392^{a}	276^{b}	275^{b}	197^{b}	196^{b}	267^{b}	218^{b}	14.8	.0408
Methionine	20	24	32	27	35	27	26	1.8	NS
Cystine	12	13	15	14	12	10	10	0.9	NS
Isoleucine	174	120	141	109	103	125	111	6.4	NS
Leucine	277	209	269	228	236	277	244	11.4	NS
Phenylalanine	118^{a}	82^{b}	62^{bc}	25^{d}	23^{d}	44^{cd}	15^{d}	7.1	.0001
Tryptophan	ND	ND	ND	21^{c}	28^{bc}	45^{ab}	57^{a}	4.1	.0001
Ornithine	132	93	131	100	86	115	101	5.3	NS
Lysine	551	444	573	487	442	462	513	20.3	NS

^{a,b,c,d} denote significance by Tukey's multiple comparison test ($p<0.05$);

NS = not significant ($p>0.05$); ND = not detectable; Trp detection limit:~10 μmol/L

decreased from 118 to 82 μmol/L as tryptophan intake increased from 0.025 to 0.05 g/kg/d ($p<0.05$), and dropped again to 25 μmol/L at 0.15 g tryptophan/kg/d ($p<0.05$). Plasma phenylalanine concentrations were similar for piglets fed 0.15-0.40 g tryptophan/kg/d. Tyrosine concentrations in plasma significantly declined ($p<0.05$) between tryptophan levels of 0.025-0.10 g/kg/d, and remained stable among tryptophan intakes of 0.10-0.40 g/kg/d. Hydroxyproline concentrations increased significantly from 65 to 92 μmol/L for tryptophan intakes of 0.025 to 0.05 g/kg/d, respectively, and increased again to 119 μmol/L at tryptophan level of 0.15 g/kg/d ($p<0.05$). Plasma hydroxyproline levels were not significantly different for piglets given 0.15-0.40 g tryptophan/kg/d. Both glutamine and taurine concentrations in plasma significantly decreased as tryptophan intakes increased from 0.025 to 0.10 g/kg/d, and remained similar for tryptophan levels of 0.10-0.40 g/kg/d. Plasma valine concentrations dropped as tryptophan intakes increased from 0.025 to 0.05 g/kg/d, and valine levels were constant in piglets receiving 0.05-0.40 g tryptophan/kg/d. Lastly, plasma tryptophan concentrations were below detection for dietary tryptophan levels 0.025-0.10 g/kg/d. Plasma tryptophan concentrations increased ($p<0.05$) as the intake of tryptophan rose from 0.15-0.40 g/kg/d.

3.4.2.2 Parenterally Fed Piglets

Plasma phenylalanine concentrations were not significantly affected by graded tryptophan intakes in parenterally fed piglets (Table 3.6). As dietary tryptophan intake increased from 0.025 to 0.05 g/kg/d, plasma tyrosine concentrations decreased from 328

Table 3.6 Plasma Amino Acid Concentrations of Parenterally Fed Piglets

	Tryptophan Intake (g/kg/d)								
Amino Acid (μmol/L)	0.025	0.05	0.10	0.15	0.20	0.30	0.40	SE	ANOVA p value
Aspartate	61	47	39	30	39	34	26	3.1	NS
Glutamate	325^{a}	225^{b}	234^{b}	149^{c}	147^{c}	167^{bc}	136^{c}	16.0	0.0017
Hydroxyproline	44	50	60	63	57	58	80	4.4	NS
Serine	535	575	337	277	232	290	305	36.0	NS
Glycine	1527	1467	682	669	459	569	751	107.0	NS
Glutamine	418^{a}	347^{a}	185^{b}	95^{b}	154^{b}	83^{b}	115^{b}	27.1	.0013
Taurine	317^{a}	220^{b}	151^{c}	129^{c}	109^{c}	137^{c}	132^{c}	15.3	.0003
Histidine	8	4	3	3	1	0	2	1.0	NS
Citrulline	153^{a}	77^{ab}	62^{ab}	40^{ab}	33^{b}	41^{ab}	60^{ab}	11.2	.0368
Threonine	194	283	323	330	420	329	412	28.7	NS
Alanine	452	512	467	418	431	397	495	19.1	NS
Arginine	142	117	95	62	94	72	105	7.7	NS
Proline	854	772	620	561	529	452	546	35.5	NS
Tyrosine	328^{a}	195^{b}	116^{bc}	64^{c}	58^{c}	72^{c}	67^{c}	20.6	.0011
Valine	265	244	214	136	154	136	180	11.7	NS
Methionine	12	12	13	16	15	26	15	1.5	NS
Cystine	18	20	17	143	12	12	23	1.3	NS
Isoleucine	137	149	118	95	93	85	94	6.8	NS
Leucine	229	231	236	182	184	167	220	9.9	NS
Phenylalanine	153	127	82	65	67	78	75	8.2	NS
Tryptophan	ND	ND	ND	4^{c}	17^{bc}	28^{ab}	39^{a}	2.9	.0042
Ornithine	125	153	120	112	77	71	94	8.0	NS
Lysine	806	726	582	465	517	431	521	38.9	NS

^{a,b,c,d} denote significance by Tukey's multiple comparison test ($p<0.05$);
NS = not significant ($p>0.05$); ND = not detectable; Trp detection limit:~10μmol/L

to 195 μmol/L ($p<0.05$) and continued to decrease to 64 μmol/L at a dietary tryptophan level of 0.15 g/kg/d ($p<0.05$). Further graded increases in tryptophan resulted in similar plasma tyrosine concentrations. Plasma glutamate concentrations followed a similar pattern: as tryptophan intake increased from 0.025 to 0.05 g/kg/d, and from 0.05 to 0.15 g/kg/d, glutamate concentrations significantly declined ($p<0.05$) and then remained constant between dietary tryptophan levels of 0.20-0.40 g/kg/d. Glutamine concentrations dropped significantly between tryptophan levels of 0.025 and 0.10 g/kg/d and were similar between tryptophan intakes of 0.15-0.40 g/kg/d. Plasma taurine concentrations were reduced in a stepwise fashion from tryptophan intakes of 0.025 to 0.05 g/kg/d ($p<0.05$), and from tryptophan levels of 0.05-0.10 g/kg/d ($p<0.05$). Taurine concentrations were not significantly different from piglets receiving 0.10-0.40 g tryptophan/kg/d. Finally, plasma tryptophan concentrations for piglets given 0.025-0.15 g/kg/d of dietary tryptophan were below the detectable range, approximately 10 μmol/L, using HPLC. Tryptophan concentrations in plasma significantly increased as tryptophan intake increased above 0.15 g/kg/d.

3.4.3 Phenylalanine Kinetics

3.4.3.1 Enterally Fed Piglets

During the oxidation period, plateau values for breath $^{14}CO_2$ production, phenylalanine SRA and tyrosine SRA were reached within 2 hours after the initiation of a primed, constant infusion of tracer in all piglets. Data on phenylalanine kinetics for

enterally fed piglets are summarized in Table 3.7. Phenylalanine intake was not significantly different among dietary tryptophan levels ($p>0.05$). Phenylalanine SRA significantly increased from 28.2 $x10^3$ to 50.6 $x10^3$ dpm/μmol as dietary tryptophan increased from 0.025 to 0.15 g/kg/d, but then dropped at tryptophan levels of 0.20 and 0.30 g/kg/d. Phenylalanine SRA was not significantly different among the 0.15 to 0.30 g tryptophan/kg/d intake groups. The ratio of tyrosine SRA to phenylalanine SRA in plasma significantly declined as dietary tryptophan increased from 0.025-0.10 g/kg/d. The tyrosine to phenylalanine ratio was similar for piglets receiving tryptophan intakes of 0.10-0.40 g/kg/d. Although plasma tyrosine SRA, phenylalanine flux, non-oxidative disposal and release from protein breakdown differed between dietary treatment groups, there is not a clear trend present.

Phenylalanine oxidation, expressed as $^{14}CO_2$, significantly decreased from 523 $x10^3$ to 307 $x10^3$ dpm/kg/h as tryptophan intake rose from 0.025 to 0.50 g/kg/d (Figure 3.2). Values for this parameter continued to decline to 34 $x10^3$ dpm/kg/h as dietary tryptophan increased to 0.15 g/kg/d ($p<0.05$). Further increases in dietary tryptophan did not significantly affect $^{14}CO_2$ values. As tryptophan treatment levels increased from 0.025 to 0.10 g/kg/d, percentage of dose oxidized (Figure 3.3) and plasma phenylalanine oxidation (Figure 3.4) significantly decreased. Values for these two parameters remained consistent in concentration for tryptophan levels above 0.15 g/kg/d. Phenylalanine balance increased significantly when dietary tryptophan rose from 0.025-0.10 g/kg/d ($p<0.05$). Further graded increases in tryptophan intake had no significant effect on phenylalanine balance.

Table 3.7 Phenylalanine Kinetics in Enterally Fed Piglets (IV Tracer)

	Tryptophan Intake (g/kg/d)								
Parameter	0.025	0.05	0.10	0.15	0.20	0.30	0.40	SE	ANOVA p value
Corrected $V^{14}CO_2$ ($x10^3$ DPM/kg/h)	523^{a}	307^{b}	160^{bc}	34^{c}	34^{c}	43^{c}	37^{c}	37	<.0001
Plasma PheSRA ($x10^3$ DPM/umol)	28.2^{c}	29.2^{c}	32.9^{c}	50.6^{ab}	46.0^{abc}	34.8^{bc}	59.4^{a}	2.4	.0003
Plasma Tyr SRA ($x10^3$ DPM/umol)	4.6^{ab}	5.1^{a}	2.2^{bc}	2.6^{abc}	2.7^{abc}	1.2^{c}	3.1^{abc}	0.3	.0086
Tyr:Phe (%)	16.9^{a}	18.1^{a}	6.1^{b}	5.2^{b}	6.4^{b}	3.8^{b}	5.3^{b}	1.3	.0003
Phe Flux (Q) (umol/kg/h)	249.4^{a}	226.9^{ab}	189.5^{abc}	126.7^{bc}	145.6^{bc}	202.7$^{a/c}$	99.1^{c}	12.0	.0024
Phe Oxidation (E) (umol/kg/h)	20.0^{a}	10.8^{ab}	5.1^{b}	0.7^{b}	0.8^{b}	1.4^{b}	0.6^{b}	1.5	<.0001
Phe Intake (I) (umol/kg/h)	100.1	98.7	100.5	100.8	103.4	108.4	101.4	1.1	NS
Non-oxid. Losses (S=Q-E) (umol/kg/h)	229.4^{a}	216.1^{ab}	184.4^{abc}	126.1^{bc}	144.8^{abc}	201.4^{ab}	98.4^{c}	10.8	.0035
Breakdown (B=Q-I) (umol/kg/h)	149.3^{a}	128.2^{ab}	89.0^{ab}	26.0^{b}	42.2^{b}	94.3^{ab}	9.6^{ab*}	11.8	.0065
Phe Balance (I-E) (umol/kg/h)	80.1^{c}	87.9^{bc}	95.4^{ab}	100.1^{ab}	102.7^{a}	107.1^{a}	100.8^{ab}	2.0	.0003
% Dose Oxidized	7.8^{a}	4.8^{ab}	2.6^{bc}	0.5^{c}	0.5^{c}	0.6^{c}	0.7^{c}	0.6	<.0001

^{a,b,c,d} denote significance by Tukey's multiple comparison test ($p<0.05$); NS = not significant ($p>0.05$)

* n = 1

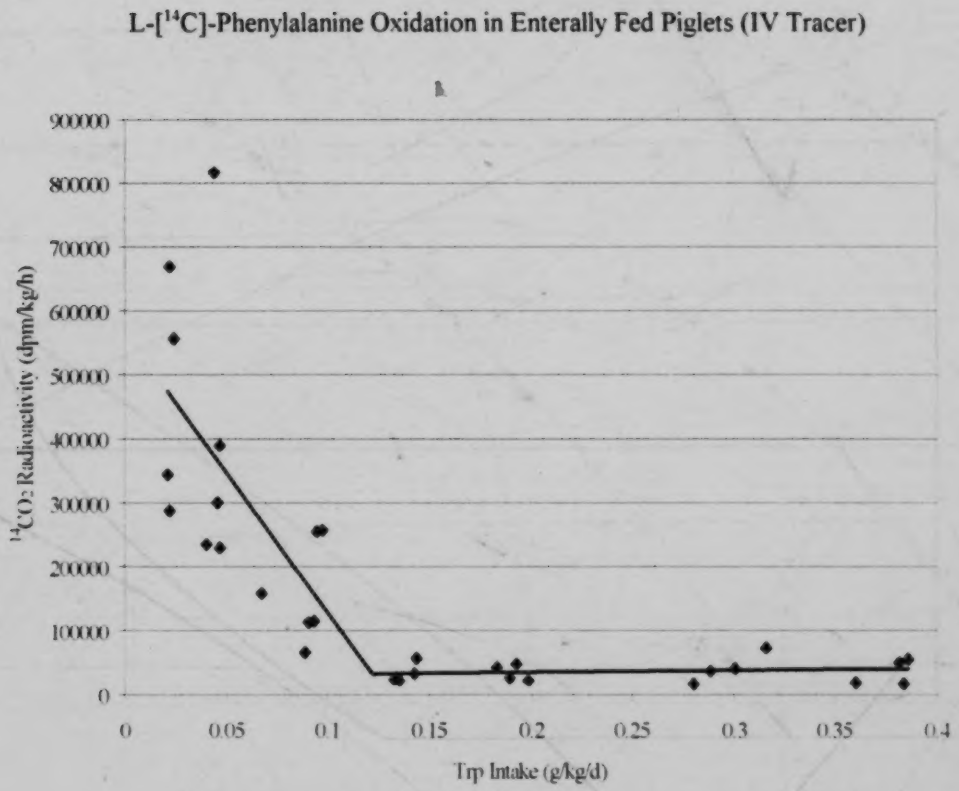

Figure 3.2 Tryptophan Requirement of Enterally Fed Piglets Receiving IV Tracer by 2-Phase Linear Regression. $^{14}CO_2$ radioactivity in collected breath of individual piglets receiving different levels of tryptophan.

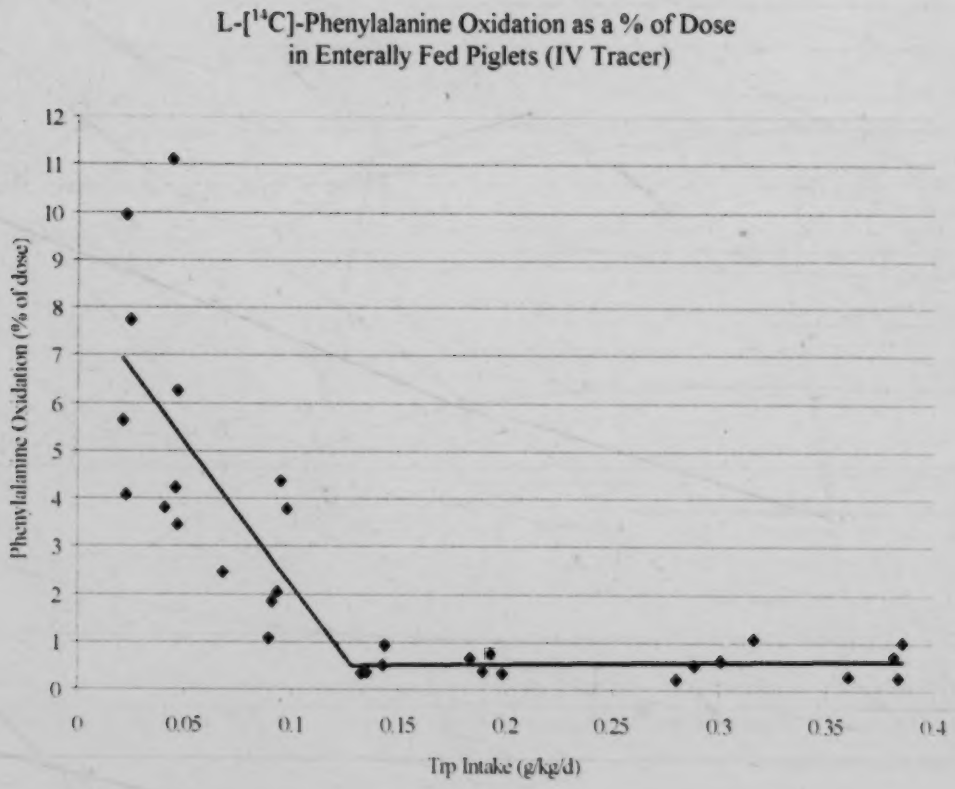

Figure 3.3 Tryptophan Requirement of Enterally Fed Piglets Receiving IV Tracer by 2-Phase Linear Regression. Phenylalanine oxidation as a % of dose in individual piglets receiving different levels of tryptophan.

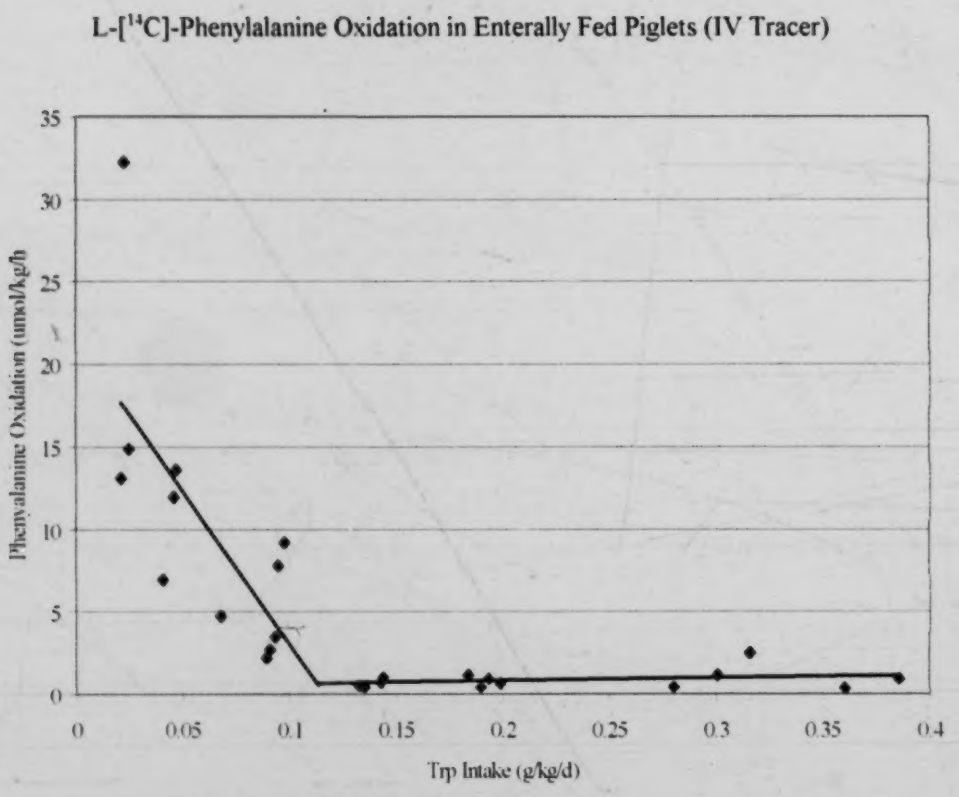

Figure 3.4 Tryptophan Requirement of Enterally Fed Piglets Receiving IV Tracer by 2-Phase Linear Regression. ^{14}C-Phenylalanine radioactivity in collected plasma of individual piglets receiving different levels of tryptophan

3.4.3.2 Parenterally Fed Piglets

During the oxidation period, plateau values for breath $^{14}CO_2$ production, phenylalanine SRA and tyrosine SRA were reached within 2 hours after the initiation of a primed, constant infusion of tracer in all piglets. Data on phenylalanine kinetics for parenterally fed piglets are summarized in Table 3.8. Plasma phenylalanine intake, flux, SRA, non-oxidative disposal, and release from protein breakdown was not significantly different among diet treatments. Plasma tyrosine SRA decreased significantly from 9.7 $x10^3$ to 3.1 $x10^3$ dpm/μmol as dietary tryptophan increased from 0.025-0.15 g/kg/d. Similarly, the ratio of tyrosine to phenylalanine SRA in plasma significantly declined from 34.2% to 9.8 % as tryptophan levels rose from 0.025 to 0.10 g/kg/d, respectively. The ratio of tyrosine to phenylalanine was similar among diet levels of 0.10-0.40 g tryptophan/kg/d.

Phenylalanine oxidation, expressed as breath $^{14}CO_2$, as calculated from plasma SRA data, or as a percentage of dose oxidized, was significantly affected by dietary intake of tryptophan. As tryptophan increased from 0.025 to 0.15 g/kg/d, breath $^{14}CO_2$ (Figure 3.5) and percentage of dose oxidized (Figure 3.6) significantly decreased ($p<0.05$). With subsequent increases in tryptophan intake from 0.15-0.40 g/kg/d, there was no change in these measures. Plasma phenylalanine oxidation also significantly decreased from 8.8 to 3.2 μmol/kg/h when dietary tryptophan rose from 0.025 to 0.10 g/kg/d, and phenylalanine oxidation was not significantly different among diet levels of 0.10-0.40 g tryptophan/kg/d (Figures 3.7). Phenylalanine balance was similar for all dietary treatments ($p>0.05$).

Table 3.8 Phenylalanine Kinetics in Parenterally Fed Piglets

	Tryptophan Intake (g/kg/d)								
Parameter	0.025	0.05	0.10	0.15	0.20	0.30	0.40	SE	ANOVA p value
Corrected $V^{14}CO_2$ ($x10^3$ DPM/kg/h)	255[a]	227[a]	115[ab]	51[b]	52[b]	50[b]	54[b]	19	.0005
Plasma Phe SRA ($x10^3$ DPM/μmol)	28.6	28.4	37.3	40.1	37.0	41.7	37.2	1.8	NS
Plasma Tyr SRA ($x10^3$ DPM/μmol)	9.7[a]	5.6[ab]	3.5[b]	3.1[b]	3.1[b]	4.0[ab]	3.1[b]	0.6	.0359
Tyr:Phe (%)	34.2[a]	20.1[ab]	9.8[b]	7.5[b]	8.5[b]	11.2[b]	9.6[b]	2.2	.0074
Phe Flux (Q) (μmol/kg/h)	259.3	283.6	205.3	183.5	198.6	201.7	200.3	12.6	NS
Phe Oxidation (E) (μmol/kg/h)	8.8[a]	8.4[a]	3.2[b]	1.2[b]	1.4[b]	1.4[b]	1.9[b]	0.7	.0001
Phe Intake (I) (μmol/kg/h)	104.0	103.1	100.4	102.5	100.8	101.7	102.9	1.2	NS
Non-oxid. Losses (S=Q-E) (μmol/kg/h)	250.5	275.2	202.1	182.3	197.2	200.2	198.4	12.2	NS
Breakdown (B=Q-I) (μmol/kg/h)	155.3	125.3	104.9	81.1	97.8	99.9	97.4	10.2	NS
Phe Balance (I-E) (μmol/kg/h)	95.2	94.8	97.2	101.2	99.4	100.3	101.0	1.1	NS
% Dose Oxidized	3.7[a]	3.2[a]	1.7[ab]	0.8[b]	0.8[b]	0.7[b]	0.8[b]	0.3	.0005

[a,b,c,d] denote significance by Tukey's multiple comparison test ($p<0.05$); NS = not significant ($p>0.05$)

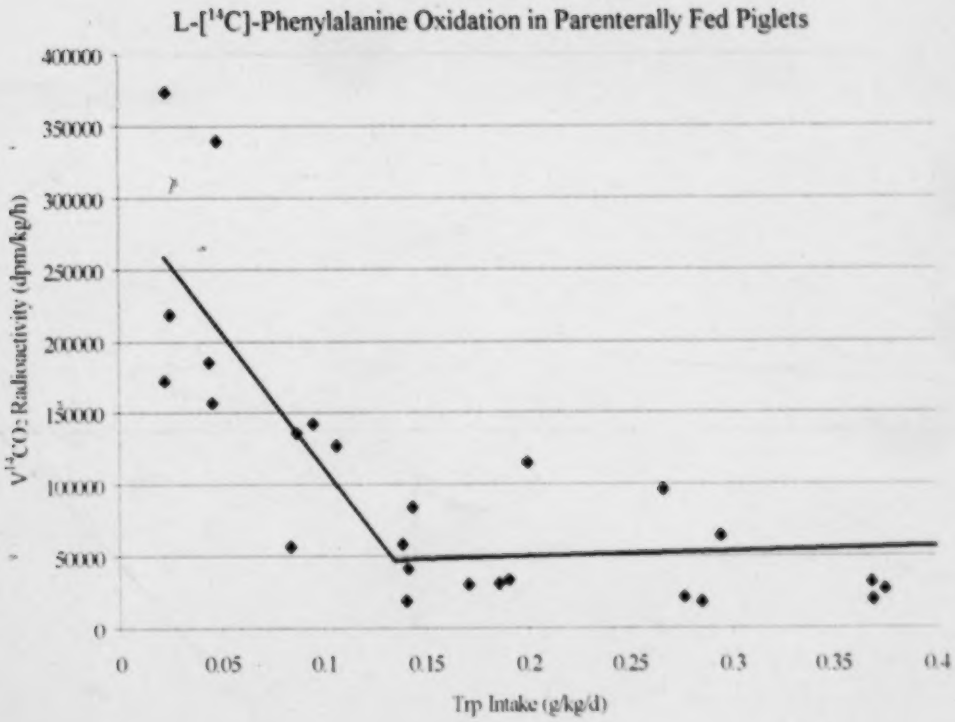

Figure 3.5 Tryptophan Requirement of Parenterally Fed Piglets by 2-Phase Linear Regression. $^{14}CO_2$ radioactivity in collected breath of individual piglets receiving different levels of tryptophan.

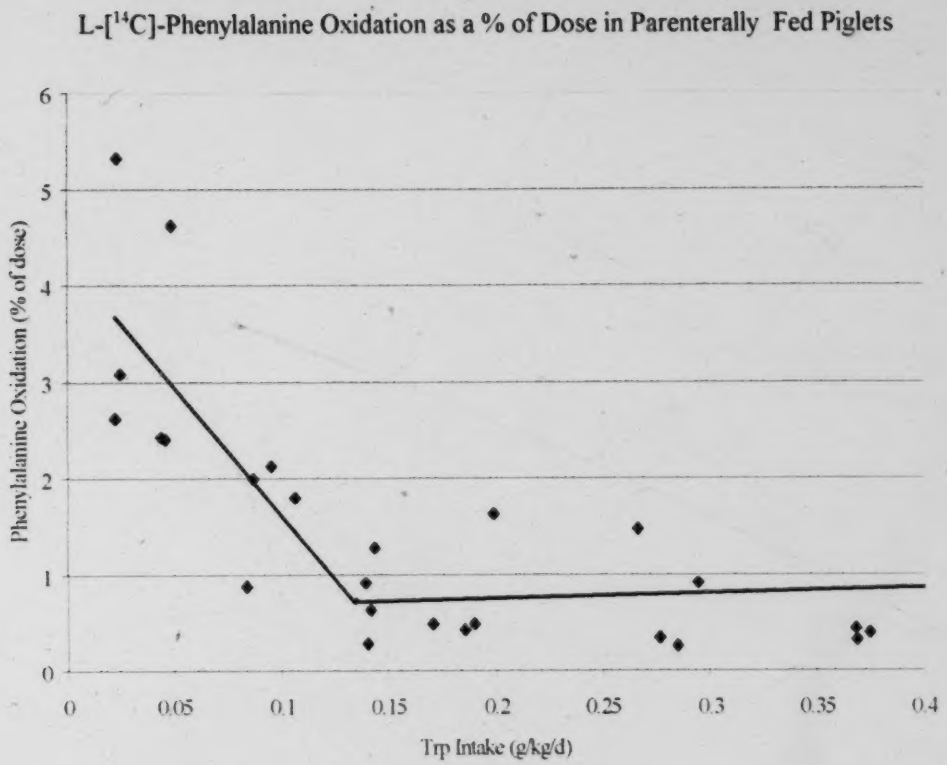

Figure 3.6 Tryptophan Requirement of Parenterally Fed Piglets by 2-Phase Linear Regression. Phenylalanine oxidation as a % of dose in individual piglets receiving different levels of tryptophan.

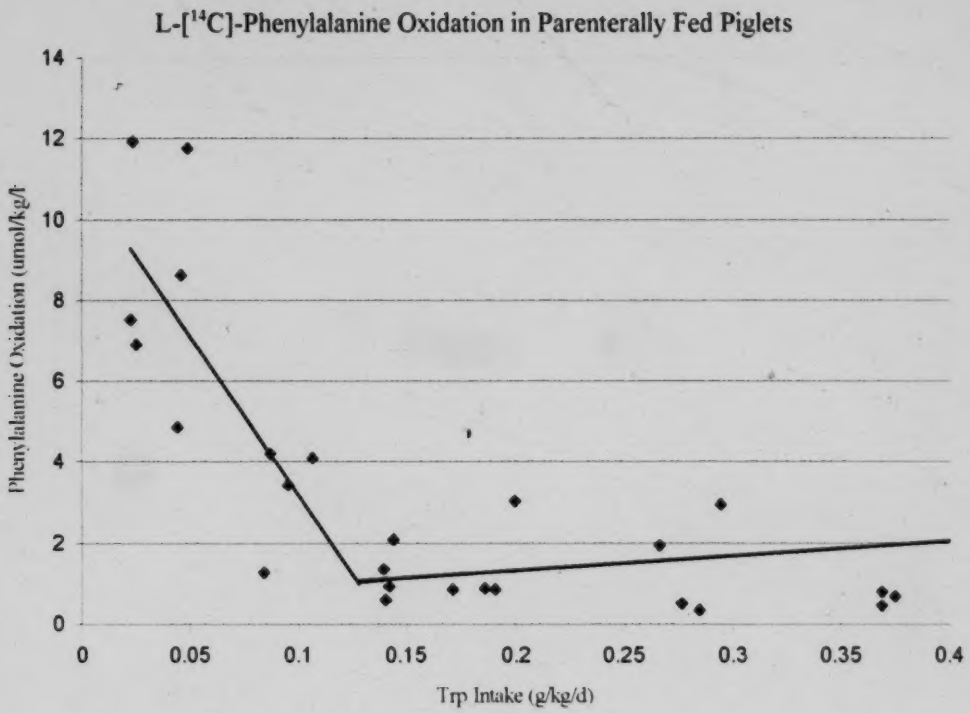

Figure 3.7 Tryptophan Requirement of Parenterally Fed Piglets by 2-Phase Linear Regression. ^{14}C-Phenylalanine radioactivity in collected plasma of individual piglets receiving different levels of tryptophan.

3.4.4 Breakpoint Analysis

To determine tryptophan requirements, a two phase linear regression crossover model was used, in which data points were partitioned between two regression lines. The data partitioning selected was based on the model that produced the highest regression coefficients and the lowest residual error. The breakpoint, or estimation of the mean tryptophan requirement, and the corresponding confidence intervals are summarized in Table 3.9 for the enterally fed animals. The breakpoint estimated by breath $^{14}CO_2$ was 0.122 g tryptophan/kg/d (CI: 0.085-0.159), by percentage of dose oxidized was 0.127 g/kg/d (CI: 0.089-0.164), by phenylalanine oxidation was 0.101 g/kg/d (CI: 0.081-0.121) and by phenylalanine balance was 0.103 g/kg/d (CI: 0.083-0.124). All of these measures yielded similar estimates due to the fact that the standard error of the estimate (SEE) for each of these measures overlapped.

The breakpoint and the corresponding confidence intervals for parenterally fed piglets are summarized in Table 3.10. The breakpoint estimated by breath $^{14}CO_2$ and phenylalanine oxidation rate was determined to be 0.142 g tryptophan/kg/d (95% CI: 0.102-0.183 and 0.107-0.178, respectively). The breakpoint determined by phenylalanine oxidation as a percentage of dose was 0.145 g tryptophan/kg/d (95% CI: 0.104-0.185). Phenylalanine balance data did not exhibit a suitable pattern for breakpoint analysis, and so was not included in this analysis. All three parameters used produced similar tryptophan requirement estimates for parenterally fed piglets.

Table 3.9 Tryptophan Requirement by Breakpoint Analysis in Enterally Fed Piglets (IV Tracer)

Parameter	Breakpoint (g/kg/d)	CI_{lower} (g/kg/d)	CI_{upper} (g/kg/d)	r^2	Root MSE	SE of Estimate
$^{14}CO_2$	0.125	0.098	0.152	0.68	121.7×10^3	0.016
Phenylalanine Oxidation	0.113	0.085	0.141	0.72	4.15	0.016
Phenylalanine Balance	0.114	0.077	0.150	0.72	5.42	0.073
% Dose Oxidized	0.127	0.089	0.164	0.71	1.66	0.022

Table 3.10 Tryptophan Requirement by Breakpoint Analysis in Parenterally Fed Piglets

Parameter	Breakpoint (g/kg/d)	CI_{lower} (g/kg/d)	CI_{upper} (g/kg/d)	r^2	Root MSE	SE of Estimate
$^{14}CO_2$	0.142	0.102	0.183	0.67	57.8×10^3	0.024
Phenylalanine Oxidation	0.142	0.107	0.178	0.71	2.01	0.021
% Dose Oxidized	0.145	0.104	0.185	0.68	0.81	0.023

3.5 DISCUSSION

Tryptophan metabolism is comprised of a complex pathway which precludes the use of direct oxidation methodology. The indicator amino acid oxidation technique has been demonstrated previously to be an appropriate method of determining amino acid requirements in enterally fed (Bertolo et al., 1998) and parenterally fed (House et al.,1997; 1998) piglets. Therefore, the IAAO method was applied to determine both enteral and parenteral tryptophan requirements for neonatal piglets.

Dietary tryptophan intake significantly influenced the plasma concentrations of several amino acids. When tryptophan intake was most limiting for protein synthesis in enterally fed piglets, the concentrations of phenylalanine, tyrosine, glutamine, taurine, and valine were significantly larger than when tryptophan was supplied at levels of 0.10 g/kg/d or greater. Similarly, parenterally fed piglets demonstrated marked elevations in glutamate, glutamine, taurine and tyrosine concentrations at dietary tryptophan intakes of less than 0.10 g/kg/d ($p<0.05$). A previous experiment detailing the lysine requirement of piglets using the IAAO method reported comparable elevations in the plasma concentrations of glutamine, valine, and phenylalanine (House et al., 1998). When tryptophan was supplied at the lowest dietary treatment levels, the body's ability to catabolize the excesses of the phenylalanine and tyrosine was likely exceeded, resulting in the observed accumulation of these amino acids in plasma. These results also suggest that the use of these indispensable amino acids were maximized when tryptophan was supplied at its requirement level. The observed rise in plasma glutamine concentrations in the

lowest tryptophan treatment groups may possibly be due to its role in the transfer of nitrogen. Ammonia (NH_4^+) groups arising from the catabolism of excess amino acids react with glutamate to form glutamine. Finally, the reason for the increase in plasma taurine concentration in the the lowest tryptophan diet groups remains unclear.

Plasma tryptophan concentrations increased from 21-57 μmol/L and from 4-39 μmol/L, respectively, when enterally and parenterally fed piglets were given 0.15-0.40 g dietary low birth weight/kg/d. At dietary tryptophan levels above 0.10 g/kg/d, enterally fed piglets demonstrated greater (although not significant, $p>0.05$) plasma tryptophan concentrations, which increased linearly and in parallel with the parenterally fed animals (Figure 3.8).

Concentrations of plasma phenylalanine and tyrosine significantly differed between the enterally and parenterally fed animals. The mean plasma phenylalanine concentration for enterally fed animals was 55.9 μmol/L compared to 92.3 μmol/L for piglets receiving parenteral diets (Figure 3.9). Tyrosine concentrations exhibited a similar pattern (Figure 3.10). Average tyrosine concentrations in plasma for the enterally and parenterally fed was 60.3 and 128.3 μmol/L, respectively. These are substantial differences, and suggest that the gut is likely utilizing phenylalanine on first pass. Stoll et al. (1998) measured the appearance of labelled amino acids in piglet portal blood following an intragastric infusion of [U-^{13}C] algal protein in combination with enteral feedings. They determined that intestinal first pass metabolism accounted for approximately 35% of phenylalanine intake. This result supports our findings, however, Stoll et al. (1998) also determined that 61% of

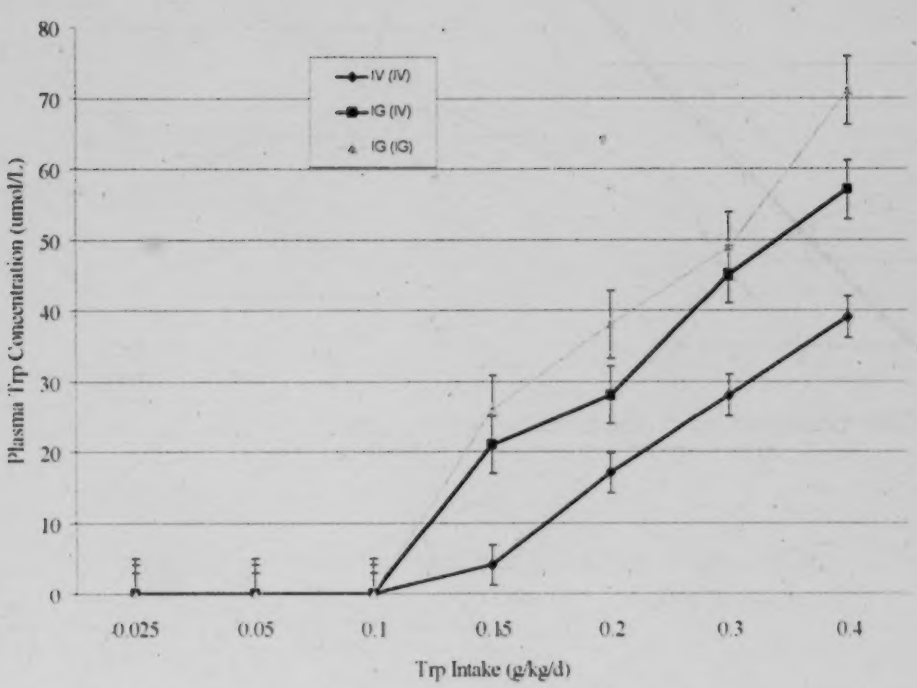

Figure 3.8 Tryptophan Concentrations in Plasma of Piglets Receiving Parenteral Diet and IV Tracer (IV (IV)), Enteral Diet and IV Tracer (IG (IV)) or Enteral Diet and Tracer (IG (IG)). Data are means ± pooled SE.

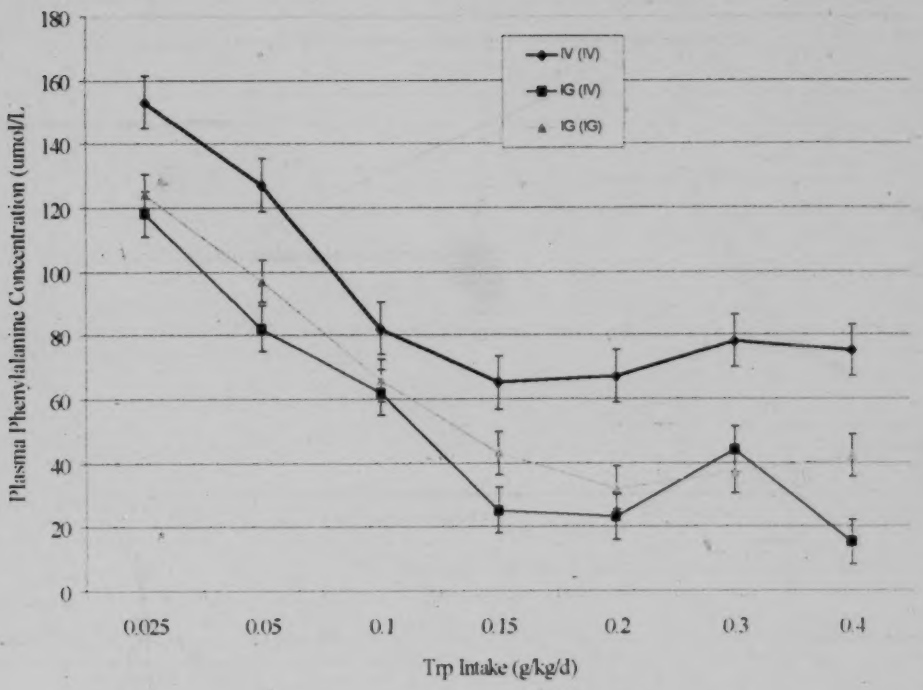

Figure 3.9 Phenylalanine Concentrations in Plasma of Piglets Receiving Parenteral Diet and Tracer (IV (IV)), Enteral Diet and IV Tracer (IG (IV)), or Enteral Diet and Tracer (IG (IG)). Data are means ± pooled SE.

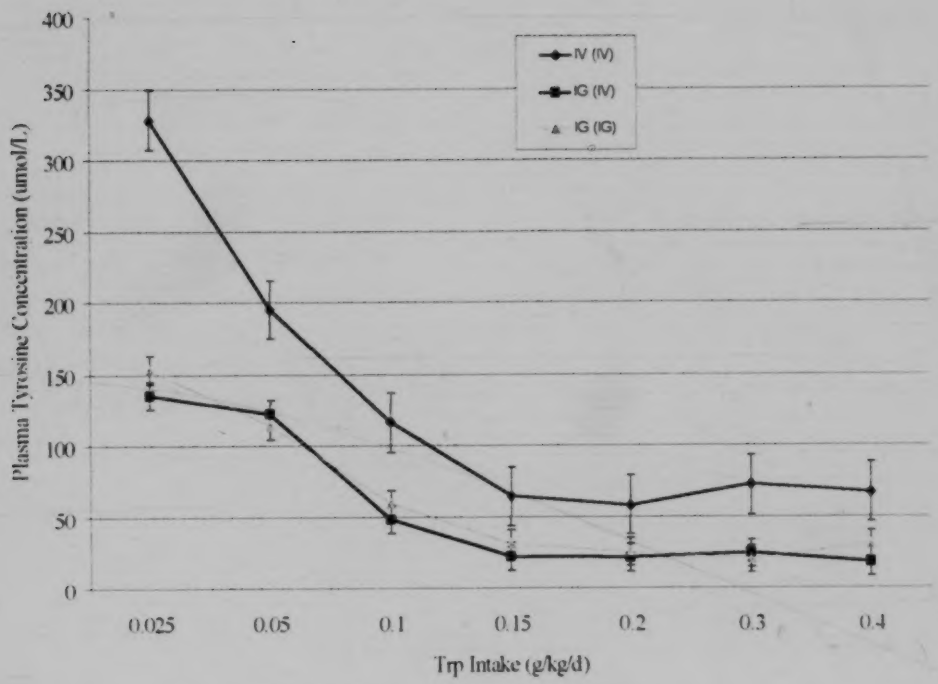

Figure 3.10 Tyrosine Concentrations in Plasma of Piglets Receiving Parenteral Diet and Tracer (IV (IV)), Enteral Diet and IV Tracer (IG (IV)), or Enteral Diet and Tracer (IG (IG)). Data are means ± pooled SE.

dietary threonine were utilized on first pass. We found that plasma threonine concentrations in enterally fed piglets were approximately twice as large as those in the parenterally fed group. It is unclear why threonine concentrations in plasma do not support the pattern seen with phenylalanine and tyrosine, however, Bertolo et al. (1999) found similar changes in amino acid concentrations in enterally compared to parenterally fed piglets. Enterally fed animals demonstrated threonine plasma concentrations that where approximately 2-fold larger than parenterally fed piglets, but this difference was not statistically significant due to large variability. It is possible that animals given parenteral diets had greater threonine oxidation than the enterally fed group.

The safe intake of phenylalanine for parenterally fed piglets has been estimated to be 0.48 g/kg/d, which is the amount provided in the elemental diets. Gut utilization of phenylalanine on first pass was witnessed in this experiment (Figure 3.9), and has been estimated to account for 35% of enterally fed labelled phenylalanine (Stoll et al., 1998), as mentioned previously. It is likely that phenylalanine was the next limiting amino acid, due to the fact that percentage of dose oxidized for animals receiving test diets containing tryptophan levels above the estimated requirement was approximately 0.5-0.8%. Thus, phenylalanine may have become limiting in enterally fed animals.

Plasma phenylalanine SRA in piglets fed enterally was significantly lower at the lowest dietary tryptophan level than at tryptophan requirement. Due to the fact that plasma phenylalanine concentrations were significantly higher in the lowest tryptophan treatment groups, it may be that the labelled phenylalanine was diluted by the additional

non-labelled phenylalanine in the plasma. Although phenylalanine SRA was not significantly different among tryptophan treatment groups for parenterally fed piglets, a similar pattern existed for these animals. In contrast, tyrosine SRA and plasma tyrosine concentrations for parenterally fed piglets was substantially greater at the lowest tryptophan intake, and then decreased and remained constant at tryptophan levels at and above requirement. It appears that there was a greater accumulation of label in the tyrosine pool, in proportion to total tyrosine plasma concentrations, compared to phenylalanine. Phenylalanine is converted to tyrosine and then is further oxidized to CO_2. The complete oxidation of tyrosine may be the rate limiting step in the catabolism of phenylalanine, more so than the conversion of phenylalanine to tyrosine. Indeed, the ratio of plasma tyrosine SRA to phenylalanine SRA was significantly greater for the lowest dietary tryptophan levels, indicating an accumulation of tracer was primarily in the tyrosine pool. The change in the combined phenylalanine and tyrosine SRA from the lowest diet level to that at requirement, however, accounted for less than 1% of the infused tracer. Consequently, it is unlikely that breakpoint estimates were affected by the observed changes in plasma amino acid concentrations and SRA.

Calculated measures of phenylalanine intake, flux, non-oxidative disposal and breakdown from protein were not significantly different among dietary tryptophan intakes for parenterally fed piglets. Previous work which used the IAAO method to determine lysine and threonine requirements in parenterally fed neonatal piglets also found no differences in these parameters over dietary treatment levels (House et al., 1998; C. Chen

MSc thesis, 1997). Although enterally fed piglets receiving different levels of dietary tryptophan demonstrated significant differences in flux, non-oxidative disposal and breakdown of phenylalanine, values for these measures did not appear to follow a clear trend when analyzed with Tukey's multiple comparison test. Phenylalanine intake did not differ among tryptophan treatment levels in enterally fed piglets, and were not different between animals receiving either route of feeding.

Phenylalanine oxidation, expressed as breath $^{14}CO_2$, calculated from plasma phenylalanine SRA, or expressed as a percentage of dose oxidized, decreased significantly when dietary tryptophan increased from insufficient levels to requirement, for both enterally and parenterally fed animals. The decline in phenylalanine oxidation demonstrated that amino acid oxidation decreased as the limiting amino acid increased, reflecting an increase in protein synthesis until the requirement for tryptophan was met. Phenylalanine balance increased along with the increase in tryptophan intake for enterally fed piglets, as expected, however, there was no change in apparent phenylalanine balance in parenterally fed animals. Phenylalanine balance was determined by the subtraction of plasma phenylalanine oxidation from dietary phenylalanine intake. Plasma phenylalanine oxidation appeared to be greater in enterally fed compared to parenterally fed piglets at the lowest dietary tryptophan level (20.0 vs 8.8 μmol/kg/h), as expected for piglets with healthy, functioning gastrointestinal tracts. It is likely that our inability to see significance in balance data for parenterally fed piglets is due to the lower level of phenylalanine oxidation rates determined for animals receiving deficient dietary tryptophan levels.

The breakpoint, or tryptophan requirement estimate, is commonly based on the model which produces the highest regression coefficient and the lowest amount of variability. Using this criteria for enterally fed piglets, plasma phenylalanine oxidation appeared to be the most appropriate model. Due to the fact that plasma phenylalanine oxidation values were calculated from phenylalanine SRA, and that phenylalanine SRA was significantly affected by dietary tryptophan intakes, it is possible that a greater amount of error would be associated with breakpoint estimates derived from plasma phenylalanine oxidation and balance data. Therefore, percentage of dose oxidized was used to determine the breakpoint for both enterally and parenterally fed piglets. The mean tryptophan requirement for enterally fed animals was 0.127 g/kg/d with a safe upper limit of 0.164 g/kg/d. The average tryptophan requirement estimate for parenterally fed piglets was 0.145 g/kg/d with a safe upper limit of 0.185 g/kg/d. The breakpoint estimates for enterally fed piglets are well within the confidence intervals and standard error of the estimate (SEE) for parenterally fed piglets (Tables 3.9, 3.10). Therefore, the enteral and parenteral tryptophan requirement in neonatal piglets appears to be similar. This finding suggests that the gut is not preferentially utilizing tryptophan for any processes other than protein synthesis. Unlike tryptophan, the parenteral threonine requirement has been found to be only ~ 45% of the mean enteral threonine requirement (Bertolo et al., 1998), and the parenteral requirement of methionine in the absence of cysteine was ~ 69% of the mean requirement in enterally fed piglets (Shoveller et al., 2000). Thus, the amino acid needs of the parenterally fed individual must be determined empirically, and cannot be estimated

based on assumptions made concerning splanchnic first pass metabolism.

The tryptophan requirements determined in the enterally and parenterally fed animals are similar to the NRC (1998) current recommendations. For 3 kg piglets, the mean tryptophan requirement was estimated at 0.153 g/kg/d. This estimate falls within the upper confidence intervals of both the enterally and parenterally fed animals in this study. The NRC (1998) recommendations for 3 kg piglets are based heavily on the work of Ball and Bayley (1984). Ball and Bayley (1984) estimated the tryptophan requirement of 2.5 kg piglets fed an enteral, semi-purified diet using the IAAO method. They determined that the young piglet requires 2 g/kg of a 240 g protein/kg diet, which is the equivalent of approximately 0.83 g tryptophan/ 100g protein. Our requirement estimates for enterally and parenterally fed piglets was 0.127 and 0.145 g tryptophan/kg/d, or approximately 0.80 and 0.92 g tryptophan/100 protein fed, respectively. Therefore, these current tryptophan requirement estimates are similar to those previously reported, and do not differ from the NRC's current recommendations.

4.0 EFFECT OF ROUTE OF ISOTOPE INFUSION ON THE TRYPTOPHAN REQUIREMENT DETERMINED BY INDICATOR AMINO ACID OXIDATION

4.1 INTRODUCTION

Amino acid oxidation is increasingly being used to determine amino acid requirements (Bertolo et al., 1998; Brunton et al., 1998). Classically, the tracer has been given IV in order to avoid splanchnic metabolism. Controversy exists, however, with regard to the comparability of requirement estimates from IV versus IG tracer administration. The issues include not only splanchnic extraction of tracer, but also isotopic dilution of plasma, and subsequent estimates of tracer kinetics which are determined using plasma enrichment estimates.

When labelled amino acids are given orally, a significant proportion of tracer is extracted by the splanchnic tissues on first pass (Matthews et al., 1993; Biolo et al., 1992, Hoerr et al., 1991; Krempf et al., 1990; Stoll et al., 1998; van Goudoever et al., 2000). For example, approximately 30-35% of gastrically fed phenylalanine was estimated to be taken up by the splanchnic bed on first pass in humans (Matthews et al., 1999) and piglets (Stoll et al., 1998). Such splanchnic extraction has implications for the apppearance of label in plasma and estimates of tracer kinetics.

The route of tracer administration also affects isotope dilution in the plasma pool. Consequently, estimates of amino acid kinetic parameters, such as plasma specific

radioactivity (SRA) and plasma tracer flux, may be different when tracers are given IG versus IV. Isotopic enrichment (or SRA) of labelled amino acids have been shown to be significantly lower and flux significantly higher in plasma of subjects receiving oral versus IV tracers (Sanchez et al., 1995; Hoerr et al., 1991; Krempf et al., 1990). These measurements are often used to calculate amino acid oxidation and other kinetic parameters and thus may potentially alter subsequent amino acid requirement estimates, which are dependent upon amino acid or label oxidation as an end point.

While several investigators have studied the effect of the route of tracer administration on amino acid kinetics (Sanchez et al., 1995; El-Khoury et al., 1998; Hoerr et al., 1991; 1993; Yu et al., 1990; 1992), no one has yet studied the effect of the route of isotope administration on an amino acid requirement estimate. In the present experiment we studied whether the route of isotope (1-^{14}C-phenylalanine) administration (IG vs. IV) would result in any change in the estimate of tryptophan requirements in enterally fed piglets. We chose to do so with tryptophan as the test amino acid, because in the previous experiment we showed that by passing the splanchnic bed did not alter the tryptophan requirement estimate, thereby avoiding potentially confounding variables.

4.2 OBJECTIVES

1) To determine the tryptophan requirement using an IG administered tracer in enterally fed piglets with the indicator amino acid oxidation method.

2) To compare estimates of tryptophan requirements and measures of phenylalanine kinetics between enterally fed piglets receiving an IG tracer (the present experiment) with enterally fed piglets receiving an IV tracer (Chapter 3).

4.3 METHODS

4.3.1 Study Design

The procedures involving enterally fed animals given IV isotope has been described previously in Chapter 3. The enterally infused piglets were assigned to dietary tryptophan intakes in a completely randomized crossover design (Figure 4.1). These experiments were designed to compare the tryptophan requirement of enterally fed piglets, using either an IG or IV infused tracer.

4.3.2 Animals and Surgical Procedures

Experimental protocols were approved by the local animal care committee. Male Yorkshire piglets (N=18), which had been sow fed for 1.5 ± 0.5 days, were obtained from Shooter's Hill Livestock (Calmar, AB) and transported to the University of Alberta. Pre-surgical and surgical procedures were conducted as described previously (see section 3.3.2). Briefly, piglets were pre-medicated with atropine, anaesthetized with ketamine and acepromazine, and maintained with halothane. Using aseptic techniques, both the stomach and the femoral vein were catheterized. Piglets were fitted with cotton jackets, and given post-surgical antibiotic as well as an intramuscular injection of analgesic (Buprenex, 0.12 mL).

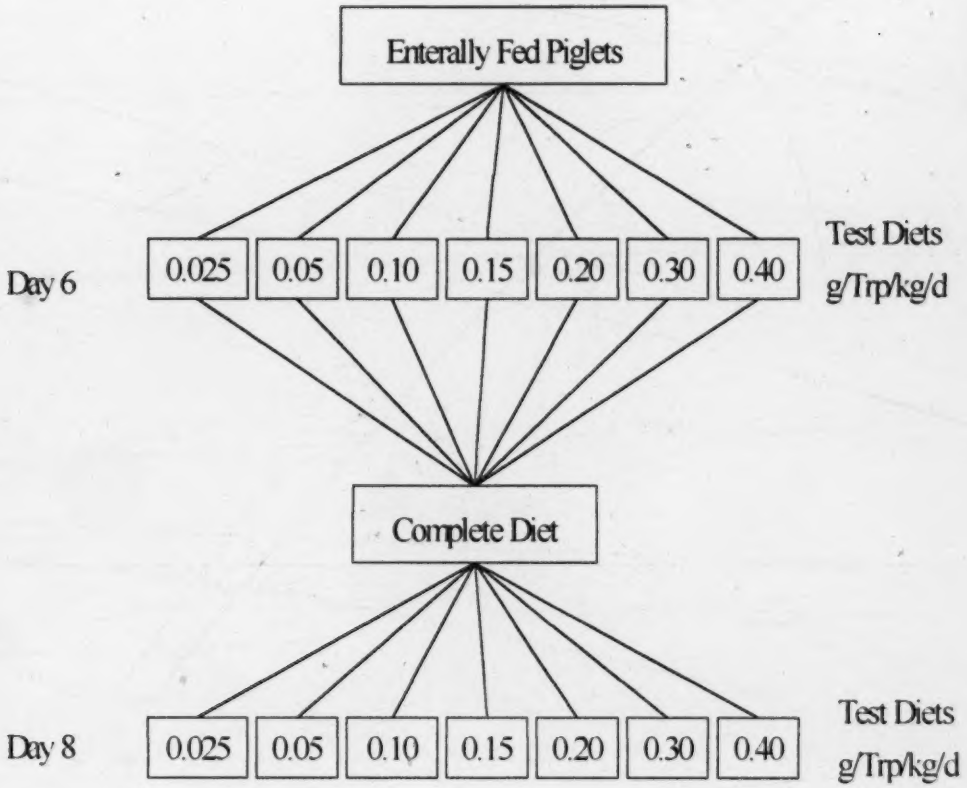

Figure 4.1 Study Design: Enterally Fed Piglets (IG versus IV Tracer).

4.3.3 Housing Conditions

Animals were housed and handled as described previously (see section 3.3.3). Piglets were housed individually, using the aforementioned tether-swivel system. Cages were arranged in groups of four so piglets maintained audio and visual contact with each other. Heat lamps were attached to the cages to maintain a temperature of ~32 °C, and light was provided between 08:00-22:00. Towels and toys were placed in the cages for environmental enrichment.

4.3.4 Diet Regimen

The enteral, complete diet used was identical to the elemental diet given to piglets in the previous experiments. The diet regimen for the piglets fed enterally was also identical to that discussed previously (see section 3.3.4). Infusion of diet began immediately following surgery, and continued until approximately 21:00 on day 5. At this time, animals were randomly assigned to receive one of 7 test diets containing one of the following levels of tryptophan: 0.025, 0.05, 0.10, 0.15, 0.20, 0.30, or 0.40 g/kg body weight/ day. To ensure all solutions were isonitrogenous, L-alanine was added in place of tryptophan when necessary. After the oxidation period was completed on day 6, piglets were returned to their cages and infusion of the complete diet was resumed. At approximately 21:00 on day 7, piglets were again randomly assigned to one of the 7 test diets. A second oxidation period was completed on day 8 (see Figure 4.1).

4.3.5 Oxidation Periods

Oxidation studies were conducted as described previously (see section 3.3.5). On days 6 and 8, animals were transferred to covered plexiglass boxes. After a 30 minute acclimation period, phenylalanine flux and oxidation was determined by a primed (5 μCi/kg), constant (3.5 μCi/kg/h) infusion of a tracer solution containing 2.5 μCi/mL of L-[1-^{14}C]- phenylalanine. Piglets were infused with a tracer either via the gastric catheter or the jugular vein. Air was drawn from the boxes by pump and the total amount of $^{14}CO_2$ expired was trapped in a series of gas washing bottles containing CO_2 absorber (ethanolamine and ethylene glycol monomethylether, 1:2, v/v). Blood samples (1.5 mL) were taken immediately prior to, and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 hours after initiation of label infusion. Blood samples were centrifuged, the plasma was collected and then stored at -20^{0} C until later analysis for phenylalanine specific radioactivity. On day 8, the second oxidation period, blood samples were taken at 60 and 30 minutes prior to label infusion to measure background radioactivity from the infusion on day 6. In addition, background breath samples were collected for 30 minutes at 45 and 15 minutes prior to label infusion. These blood and breath samples were used to correct the results of the second oxidation period for residual radioactivity. Immediately following the oxidation period on day 8, animals were given a lethal dose (750 mg) of sodium pentobarbital through the venous sampling line.

4.3.6 Analytical Procedures & Calculations

Breath and blood analysis, as well as sample calculations were completed as described in the previous chapter (see sections 3.3.6 and 3.3.7).

4.3.7 Statistical Analysis

These experiments consisted of a completely randomized crossover design, with dietary tryptophan level acting as the main treatment effect. Breakpoint analysis, with a two-phase linear crossover model, was used to determine mean tryptophan requirements and 95% confidence intervals (see Appendix). Differences among dietary treatments with respect to day of oxidation, initial weight, final weight, and average daily gain were examined using analysis of variance (ANOVA). In addition, the effects of dietary tryptophan treatments, day of oxidation and body weight on phenylalanine oxidation (expressed as % dose oxidized) were examined using ANOVA. Tukey's multiple comparison tests were used to compare plasma amino acid concentrations and estimates of amino acid kinetics between dietary treatment groups. Finally, ANOVAs were used to compare all of these parameters between piglets given IG vs IV tracers (SAS programming system).

4.4 RESULTS

4.4.1 Weight Changes

Initial weight, final weight and average daily gain were similar among dietary tryptophan intake groups and between enterally fed piglets given either IG or IV isotope infusion. Enterally fed piglets receiving IG and IV tracer had average initial weights of 1.59 and 1.55 kg (SD: ± 0.15 and ± 0.16 kg) respectively. Mean final weight at oxidation (day 8) was 2.91 and 2.74 kg (SD: ± 0.26 and ± 0.29 kg) for piglets receiving enteral diets with IG and IV tracer. The average daily gain for enterally fed piglets given IG tracer was 0.18 kg (SD: ± 0.03 kg) and was 0.17 kg (SD: ± 0.04 kg) for enterally fed piglets given IV tracer. Finally, none of the body weight parameters measured, or day of oxidation significantly affected phenylalanine oxidation (when expressed as % of dose oxidized).

4.4.2 Plasma Amino Acids

4.4.2.1 Enterally Fed Piglets (with IG Tracer)

Phenylalanine concentrations in plasma were significantly affected by dietary tryptophan intakes in enterally fed piglets receiving IG infused tracer (Table 4.1). Phenylalanine concentrations decreased significantly from 124 to 66 μmol/L as dietary tryptophan intake increased from 0.025 to 0.10 g/kg/d. Tyrosine concentrations also declined from 153 to 59 μmol/L as tryptophan levels rose from 0.025 to 0.10 g/kg/d. Both plasma phenylalanine and tyrosine concentrations were not significantly different for

piglets receiving 0.10-0.40 g tryptophan/kg/d. Hydroxyproline levels in plasma increased significantly, when dietary tryptophan increased from 0.025-0.15 g/kg/d, and then remained constant for tryptophan intakes of 0.15-0.40 g/kg/d. Plasma asparagine and taurine concentrations significantly declined between tryptophan levels of 0.025- 0.15 g/kg/d, and were not significantly different between 0.15-0.40 g tryptophan/kg/d. Glutamine concentrations dropped from 473 to 277 μmol/L as tryptophan intake increased from 0.025-0.10 g/kg/d ($p<0.05$), and decreased further to 137 μmol/L at a dietary tryptophan level of 0.15 g/kg/d ($p<0.05$). Further increases in tryptophan intake did not significantly affect plasma glutamine concentrations. Finally, plasma tryptophan concentrations were below detection (~ 10 μmol/L) for dietary tryptophan levels 0.025-0.10 g/kg/d and significantly increased to 26 μmol/L at an intake of 0.15 g tryptophan/kg/d. Plasma tryptophan concentrations continued to increase ($p<0.05$) as the intake of tryptophan rose above 0.15 g/kg/d.

4.4.2.2 Enterally Fed Piglets (with IV Tracer)

Plasma amino acid concentrations of piglets fed enterally in combination with IV administered tracer was discussed previously, in section 3.4.2.1. Several plasma amino acid concentrations were significantly influenced by graded tryptophan intakes in enterally fed piglets (Table 4.2). Phenylalanine concentrations decreased from 118 to 82 μmol/L as tryptophan intake increased from 0.025 to 0.05 g/kg/d ($p<0.05$), and dropped again to 25 μmol/L at 0.15 g tryptophan/kg/d ($p<0.05$). Plasma phenylalanine concentrations were

Table 4.1 Plasma Amino Acid Concentrations of Enterally Fed Piglets (IG Tracer)

	Tryptophan Intake (g/kg/d)								
Amino Acid (μmol/L)	0.025	0.05	0.10	0.15	0.20	0.30	0.40	SE	ANOVA p value
Aspartate	20	18	21	27	22	22	18	1.4	NS
Glutamate	212	190	237	202	245	222	188	17.0	NS
Hydroxyproline	88^{b}	81^{b}	99^{b}	136^{a}	132^{a}	131^{a}	131^{a}	4.6	.0015
Serine	341	340	332	318	322	304	304	13.8	NS
Asparagine	44^{a}	38^{ab}	30^{abc}	15^{c}	19^{c}	23^{bc}	24^{bc}	2.2	.0075
Glycine	1136	1097	1144	1466	1350	1340	1100	89.9	NS
Glutamine	473^{a}	416^{a}	277^{b}	137^{c}	192^{bc}	184^{bc}	174^{bc}	24.3	.0001
Taurine	284^{a}	263^{ab}	189^{abc}	158^{bc}	133^{c}	166^{abc}	157^{bc}	13.7	.0267
Histidine	84	68	53	56	53	48	52	3.8	NS
Citrulline	114	106	92	79	92	81	87	4.3	NS
Threonine	518	581	805	783	1215	1034	911	62.2	NS
Alanine	561	536	681	929	931	899	939	39.2	NS
Arginine	181	164	145	188	121	139	174	7.1	NS
Proline	723	585	612	614	597	556	655	28.5	NS
Tyrosine	153^{a}	114^{a}	59^{b}	31^{b}	25^{b}	20^{b}	30^{b}	9.6	.0002
Valine	406	334	295	245	226	229	248	14.1	.NS
Methionine	15	16	17	26	28	24	26	1.3	NS
Cystine	11	10	11	12	9	9	11	0.5	NS
Isoleucine	186	157	151	130	117	117	137	5.9	NS
Leucine	335	291	293	303	260	264	302	9.3	NS
Phenylalanine	124^{a}	97^{ab}	66^{bc}	43^{c}	32^{c}	37^{c}	42^{c}	6.6	.0008
Tryptophan	ND	ND	ND	26^{b}	38^{b}	49^{ab}	71^{a}	4.8	.0001
Ornithine	158	159	174	104	121	123	145	7.2	NS
Lysine	703	644	535	593	551	582	533	24.9	NS

^{a,b,c,d} denote significance by Tukey's multiple comparison test ($p<0.05$);
NS = not significant ($p>0.05$); ND = not detectable; Trp detection limit:~10 μmol/L

Table 4.2 Plasma Amino Acid Concentrations of Enterally Fed Piglets (IV Tracer)

	Tryptophan Intake (g/kg/d)								
Amino Acid (μmol/L)	0.025	0.05	0.10	0.15	0.20	0.30	0.40	SE	ANOVA p value
Aspartate	25	17	19	18	15	28	12	1.9	NS
Glutamate	207	140	208	154	166	288	128	16.5	NS
Hydroxyproline	65^{cd}	64^{d}	92^{b}	119^{a}	95^{ab}	91^{abc}	99^{ab}	4.3	.0011
Serine	306	235	263	223	193	277	201	17.7	NS
Asparagine	51	41	20	18	17	19	16	3.6	NS
Glycine	979	746	1098	1159	745	985	762	84.0	NS
Glutamine	549^{a}	447^{ab}	213^{b}	177^{b}	161^{b}	223^{b}	172^{b}	35.2	.0177
Taurine	277^{a}	204^{ab}	148^{bc}	135^{bc}	100^{c}	136^{bc}	136^{bc}	12.5	.0123
Histidine	87	51	43	30	42	62	39	5.0	NS
Citrulline	119^{a}	104^{ab}	85^{abc}	89^{abc}	57^{c}	64^{bc}	71^{bc}	6.1	.0131
Threonine	495	439	817	830	856	872	883	62.3	NS
Alanine	583^{bc}	431^{c}	655^{bc}	758^{abc}	800^{ab}	1022^{a}	784^{ab}	44.8	.0012
Arginine	149	107	158	149	135	95	141	9.1	NS
Proline	739	480	546	537	474	717	583	35.0	NS
Tyrosine	135^{a}	122^{a}	48^{b}	22^{b}	21^{b}	24^{b}	18^{b}	9.7	.0006
Valine	392^{a}	276^{b}	275^{b}	197^{b}	196^{b}	267^{b}	218^{b}	14.8	.0408
Methionine	20	24	32	27	35	27	26	1.8	NS
Cystine	12	13	15	14	12	10	10	0.9	NS
Isoleucine	174	120	141	109	103	125	111	6.4	NS
Leucine	277	209	269	228	236	277	244	11.4	NS
Phenylalanine	118^{a}	82^{b}	62^{bc}	25^{d}	23^{d}	44^{cd}	15^{d}	7.1	.0001
Tryptophan	ND	ND	ND	21^{c}	28^{bc}	45^{ab}	57^{a}	4.1	.0001
Ornithine	132	93	131	100	86	115	101	5.3	NS
Lysine	551	444	573	487	442	462	513	20.3	NS

^{a,b,c,d} denote significance by Tukey's multiple comparison test ($p<0.05$);

NS = not significant ($p>0.05$); ND = not detectable; Trp detection limit:~10 μmol/L

similar for piglets fed 0.15-0.40 g tryptophan/kg/d. Tyrosine concentrations in plasma significantly declined ($p<0.05$) between tryptophan levels of 0.025-0.10 g/kg/d, and remained stable among tryptophan intakes of 0.10-0.40 g/kg/d. Hydroxyproline concentrations increased significantly from 65 to 92 μmol/L for tryptophan intakes of 0.025 to 0.05 g/kg/d, respectively, and increased again to 119 μmol/L at tryptophan level of 0.15 g/kg/d ($p<0.05$). Plasma hydroxyproline levels were not significantly different for piglets given 0.15-0.40 g tryptophan/kg/d. Both glutamine and taurine concentrations in plasma significantly decreased as tryptophan intakes increased from 0.025 to 0.10 g/kg/d, and remained similar for tryptophan levels of 0.10-0.40 g/kg/d. Plasma valine concentrations dropped as tryptophan intakes increased from 0.025 to 0.05 g/kg/d, and valine levels were constant in piglets receiving 0.05-0.40 g tryptophan/kg/d. Lastly, plasma tryptophan concentrations were below detection for dietary tryptophan levels 0.025-0.10 g/kg/d. Plasma tryptophan concentrations increased ($p<0.05$) as the intake of tryptophan rose from 0.15-0.40 g/kg/d.

4.4.3 Phenylalanine Kinetics

4.4.3.1 Enterally Fed Piglets (with IG Tracer)

During the oxidation period, plateau values for breath $^{14}CO_2$ production, phenylalanine SRA and tyrosine SRA were reached within 2 hours after the initiation of tracer infusion in all piglets. Phenylalanine intake and tyrosine SRA was not significantly different among diet treatments (Table 4.3). Phenylalanine SRA, however, significantly

Table 4.3 Phenylalanine Kinetics in Enterally Fed Piglets (IG Tracer)

	Tryptophan Intake (g/kg/d)								
Parameter	0.025	0.05	0.10	0.15	0.20	0.30	0.40	SE	ANOVA p value
Corrected $V^{14}CO_2$ ($x10^3$ DPM/kg/h)	317^{ab}	421^{a}	111^{b}	52^{b}	54^{b}	77^{b}	57^{b}	34	.0004
Plasma PheSRA ($x10^3$ DPM/μmol)	22.5^{bc}	20.7^{c}	24.7^{bc}	42.4^{a}	39.9^{a}	35.3^{ab}	37.3^{a}	1.9	.0001
Plasma Tyr SRA ($x10^3$ DPM/μmol)	4.3	2.9	1.2	4.0	2.6	1.0	2.9	0.4	NS
Tyr:Phe	18.5^{a}	14.0^{ab}	4.9^{b}	8.3^{ab}	6.0^{ab}	2.7^{b}	7.2^{ab}	1.5	.0130
Phe Flux (Q) (μmol/kg/h)	290.1^{ab}	324.7^{a}	281.2^{ab}	183.0^{b}	187.5^{b}	227.8^{ab}	205.5^{b}	13.4	.0034
Phe Oxidation (E) (μmol/kg/h)	14.2^{ab}	20.2^{a}	4.5^{bc}	1.3^{c}	1.4^{c}	2.0^{bc}	1.4^{c}	1.6	.0001
Phe Intake (I) (μmol/kg/h)	100.3	102.8	102.8	102.6	103.9	102.1	101.0	0.6	NS
Non-oxid. Losses (S=Q-E) (μmol/kg/h)	275.8^{ab}	304.5^{a}	276.7^{ab}	181.7^{b}	186.1^{b}	225.8^{ab}	204.1^{ab}	12.6	.0123
Breakdown (B=Q-I) (μmol/kg/h)	189.7^{ab}	221.9^{a}	178.4^{ab}	80.4^{b}	83.6^{b}	125.7^{ab}	104.5^{ab}	13.5	.0035
Phe Balance (I-E) (μmol/kg/h)	86.1^{bc}	82.7^{c}	98.3^{ab}	101.3^{a}	102.5^{a}	100.1^{ab}	99.6^{ab}	1.8	.0003
% Dose Oxidized	4.9^{ab}	6.3^{a}	1.6^{bc}	0.7^{c}	0.7^{c}	1.1^{bc}	0.9^{bc}	0.5	.0001

^{a,b,c,d} denote significance by Tukey's multiple comparison test ($p<0.05$);
NS = not significant ($p>0.05$)

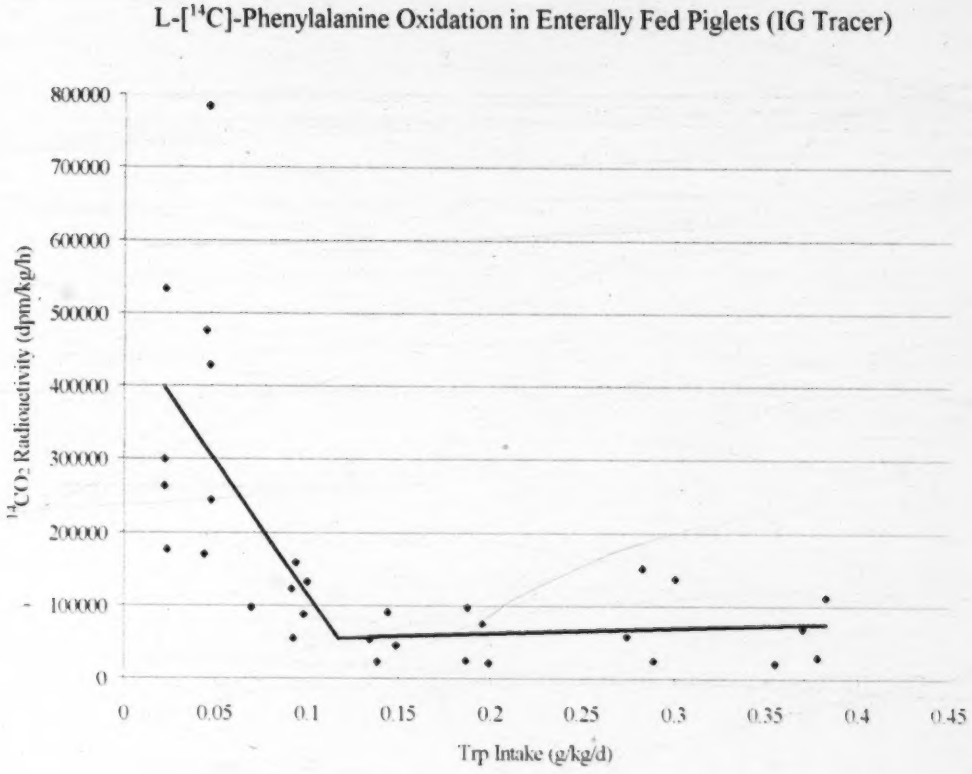

Figure 4.2 Tryptophan Requirement of Enterally Fed Piglets Receiving IG Tracer by 2-Phase Linear Regression. $^{14}CO_2$ radioactivity in collected breath of individual piglets receiving different levels of tryptophan.

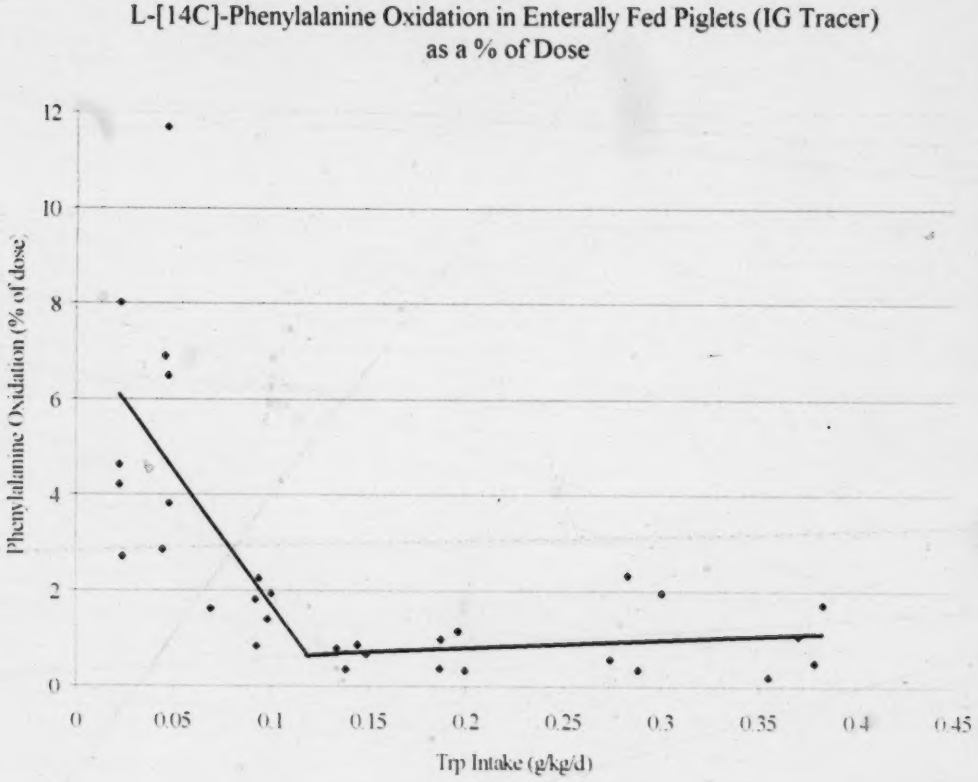

Figure 4.3 Tryptophan Requirement of Enterally Fed Piglets Receiving IG Tracer by 2-Phase Linear Regression. Phenylalanine oxidation as a % of dose in individual piglets receiving different levels of tryptophan.

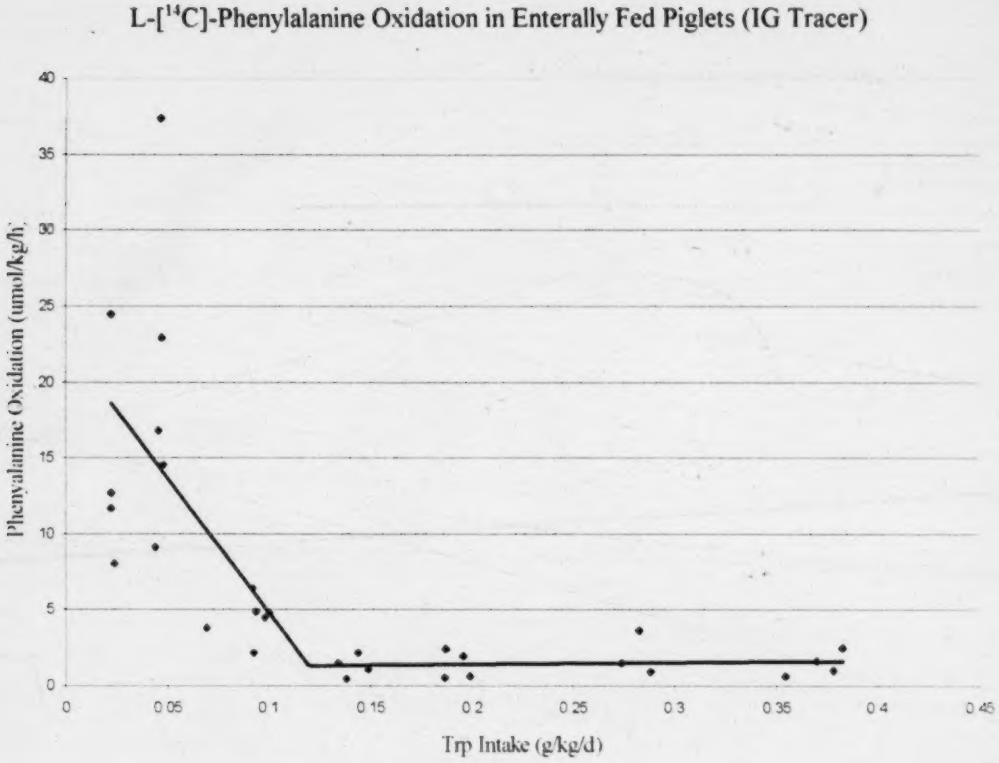

Figure 4.4 Tryptophan Requirement of Enterally Fed Piglets Receiving IG Tracer by 2-Phase Linear Regression. ^{14}C-Phenylalanine radioactivity in collected plasma of individual piglets receiving different levels of tryptophan.

increased from 22.5 $x10^3$ to 42.4 $x10^3$ dpm/μmol as dietary tryptophan increased from 0.025 to 0.15 g/kg/d. Additional intake of tryptophan above 0.15 g/kg/d did not result in any significant changes in plasma phenylalanine SRA ($p>0.05$). Although the ratio of plasma tyrosine SRA to phenylalanine SRA, phenylalanine flux, non-oxidative disposal and release from protein breakdown differed between diet treatment groups, there is not a clear trend present.

Phenylalanine oxidation, expressed as $^{14}CO_2$, significantly decreased from 421 $x10^3$ to 111 $x10^3$ dpm/kg/h as dietary tryptophan rose from 0.05 to 0.10 g/kg/d (Figure 4.2). Further graded increases in tryptophan resulted in similar $^{14}CO_2$ values ($p>0.05$). Percentage of dose oxidized (Figure 4.3), plasma phenylalanine oxidation (Figure 4.4), and phenylalanine balance significantly decreased as dietary tryptophan increased from 0.05 to 0.10 g/kg/d, and was not significantly different among diet levels of 0.10-0.40 g tryptophan/kg/d.

4.4.3.2 Enterally Fed Piglets (with IV Tracer)

Plasma phenylalanine kinetics for enterally fed piglets receiving IV tracer has been discussed previously in section 3.4.3.1. During the oxidation period, plateau values for breath $^{14}CO_2$ production, phenylalanine SRA and tyrosine SRA were reached within 2 hours after the initiation of a primed, constant infusion of tracer in all piglets. Data on phenylalanine kinetics for enterally fed piglets are summarized in Table 4.4. Phenylalanine intake was not significantly different among dietary tryptophan levels ($p>0.05$).

Phenylalanine SRA significantly increased from 28.2 $x10^3$ to 50.6 $x10^3$ dpm/μmol as dietary tryptophan increased from 0.025 to 0.15 g/kg/d, but then dropped at tryptophan levels of 0.20 and 0.30 g/kg/d. Phenylalanine SRA was not significantly different among the 0.15 to 0.30 g tryptophan/kg/d intake groups. The ratio of tyrosine SRA to phenylalanine SRA in plasma significantly declined as dietary tryptophan increased from 0.025-0.10 g/kg/d. The tyrosine to phenylalanine ratio was similar for piglets receiving tryptophan intakes of 0.10-0.40 g/kg/d. Although plasma tyrosine SRA, phenylalanine flux, non-oxidative disposal and release from protein breakdown differed between diet treatment groups, there is not a clear trend present.

Phenylalanine oxidation, expressed as $^{14}CO_2$, significantly decreased from 523 $x10^3$ to 307 $x10^3$ dpm/kg/h as tryptophan intake rose from 0.025 to 0.50 g/kg/d (Figure 4.5). Values for this parameter continued to decline to 34 $x10^3$ dpm/kg/h as dietary tryptophan increased to 0.15 g/kg/d ($p<0.05$). Further increases in dietary tryptophan did not significantly affect $^{14}CO_2$ values. As tryptophan treatment levels increased from 0.025 to 0.10 g/kg/d, percentage of dose oxidized (Figure 4.6) and plasma phenylalanine oxidation (Figure 4.7) significantly decreased. Values for these two parameters remained consistent in concentration for tryptophan levels above 0.15 g/kg/d. Phenylalanine balance increased significantly when dietary tryptophan rose from 0.025-0.10 g/kg/d ($p<0.05$). Further graded increases in tryptophan intake had no significant effect on phenylalanine balance.

Table 4.4 Phenylalanine Kinetics in Enterally Fed Piglets (IV Tracer)

	Tryptophan Intake (g/kg/d)								
Parameter	0.025	0.05	0.10	0.15	0.20	0.30	0.40	SE	ANOVA p value
Corrected $V^{14}CO_2$ ($x10^3$ DPM/kg/h)	523^{a}	307^{b}	160^{bc}	34^{c}	34^{c}	43^{c}	37^{c}	37	<.0001
Plasma PheSRA ($x10^3$ DPM/μmol)	28.2^{c}	29.2^{c}	32.9^{c}	50.6^{ab}	46.0^{abc}	34.8^{bc}	59.4^{a}	2.4	.0003
Plasma Tyr SRA ($x10^3$ DPM/μmol)	4.6^{ab}	5.1^{a}	2.2^{bc}	2.6^{abc}	2.7^{abc}	1.2^{c}	3.1^{abc}	0.3	.0086
Tyr:Phe (%)	16.9^{a}	18.1^{a}	6.1^{b}	5.2^{b}	6.4^{b}	3.8^{b}	5.3^{b}	1.3	.0003
Phe Flux (Q) (μmol/kg/h)	249.4^{a}	226.9^{ab}	189.5^{a}	126.7^{bc}	145.6^{bc}	202.7^{abc}	99.1^{c}	12.0	.0024
Phe Oxidation (E) (μmol/kg/h)	20.0^{a}	10.8^{a}	5.1^{b}	0.7^{b}	0.8^{b}	1.4^{b}	0.6^{b}	1.5	<.0001
Phe Intake (I) (μmol/kg/h)	100.1	98.7	100.5	100.8	103.4	108.4	101.4	1.1	NS
Non-oxid. Losses (S=Q-E) (μmol/kg/h)	229.4^{a}	216.1^{ab}	184.4^{a}	126.1^{bc}	144.8^{abc}	201.4^{ab}	98.4^{c}	10.8	.0035
Breakdown (B=Q-I) (μmol/kg/h)	149.3^{a}	128.2^{ab}	89.0^{ab}	26.0^{b}	42.2^{b}	94.3^{ab}	9.6^{ab*}	11.8	.0065
Phe Balance (I-E) (umol/kg/h)	80.1^{c}	87.9^{b}	95.4^{ab}	100.1^{ab}	102.7^{a}	107.1^{a}	100.8^{a}	2.0	.0003
% Dose Oxidized	7.8^{a}	4.8^{ab}	2.6^{bc}	0.5^{c}	0.5^{c}	0.6^{c}	0.7^{c}	0.6	<.0001

^{a,b,c,d} denote significance by Tukey's multiple comparison test ($p<0.05$); NS = not significant ($p>0.05$)

* n = 1

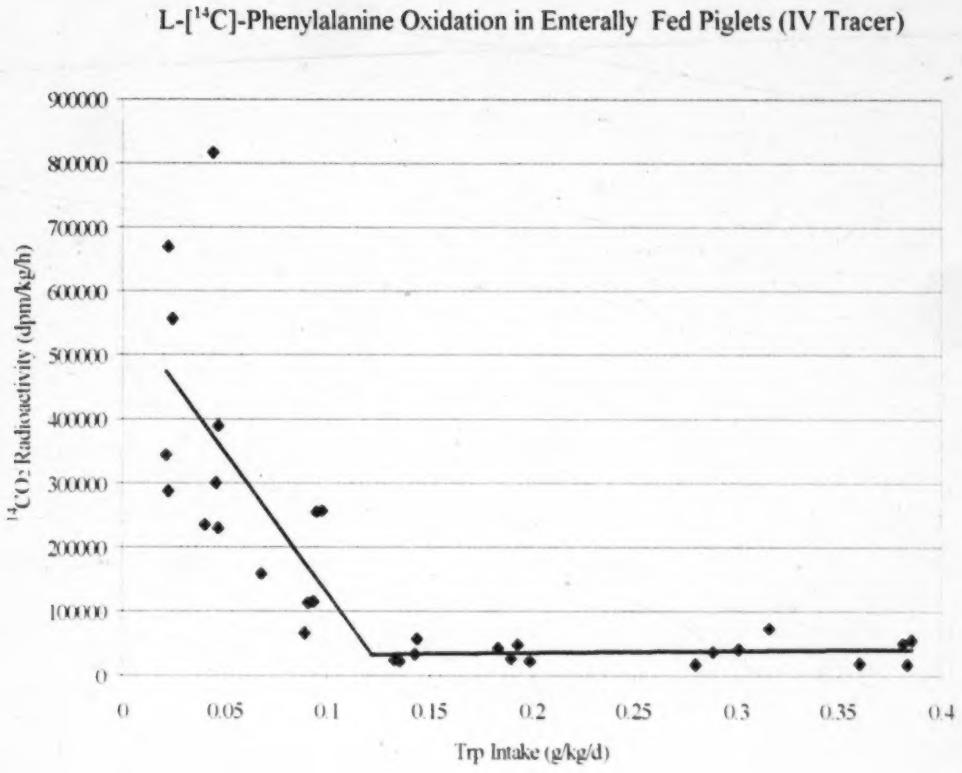

Figure 4.5 Tryptophan Requirement of Enterally Fed Piglets Receiving IV Tracer by 2-Phase Linear Regression. $^{14}CO_2$ radioactivity in collected breath of individual piglets receiving different levels of tryptophan.

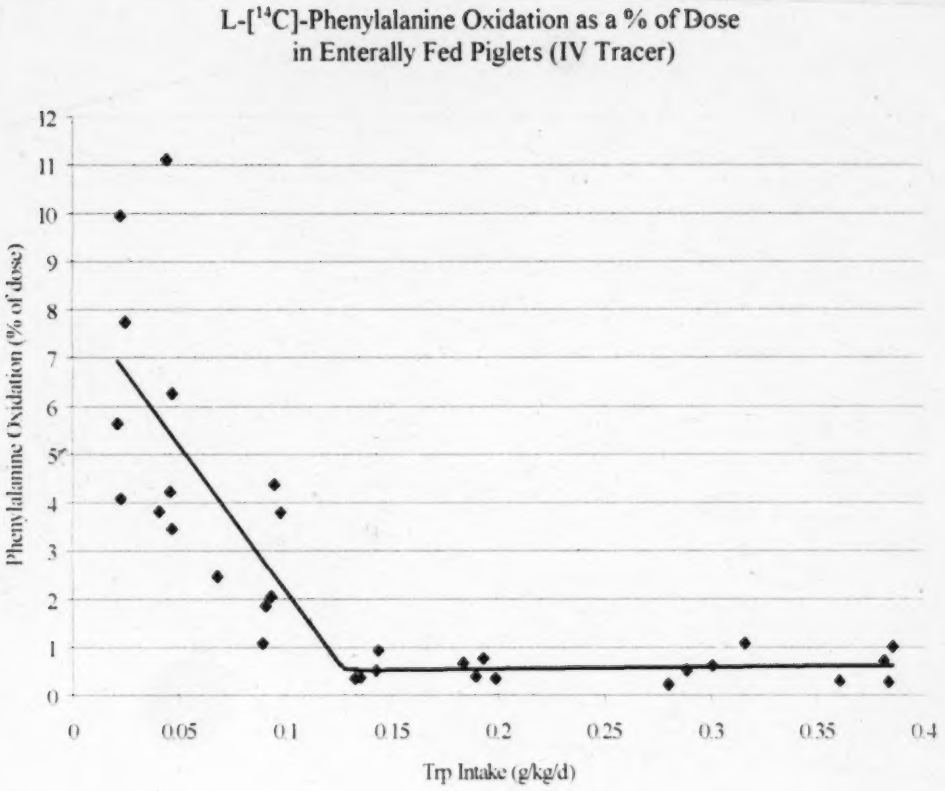

Figure 4.6 Tryptophan Requirement of Enterally Fed Piglets Receiving IV Tracer by 2-Phase Linear Regression. Phenylalanine oxidation as a % of dose in individual piglets receiving different levels of tryptophan.

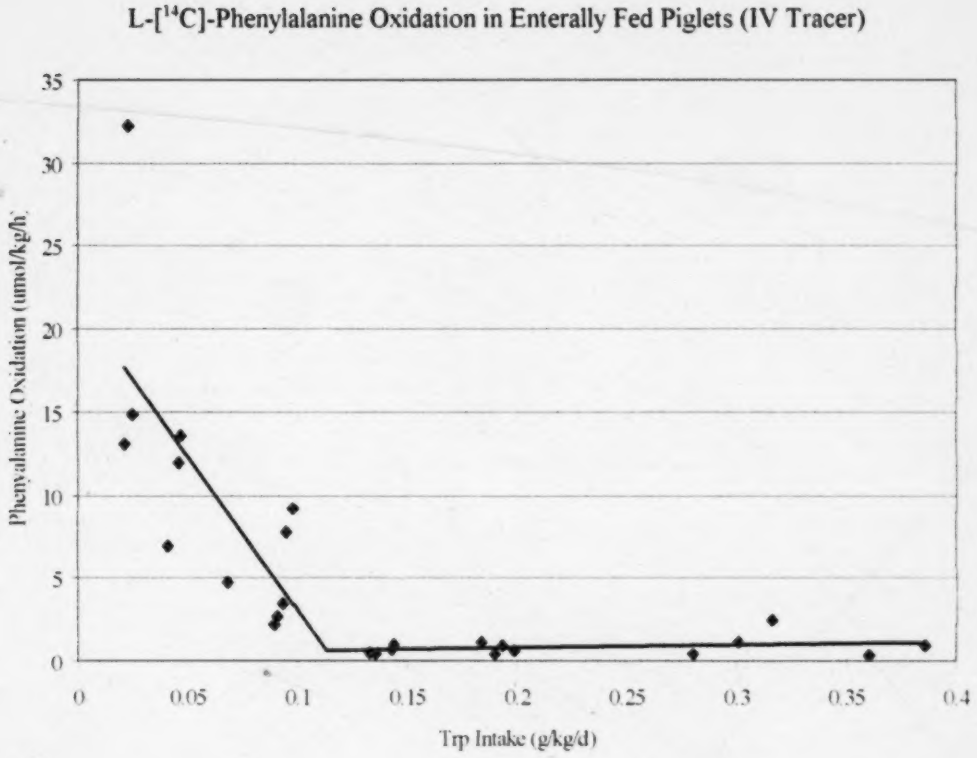

Figure 4.7 Tryptophan Requirement of Enterally Fed Piglets Receiving IV Tracer by 2-Phase Linear Regression. ^{14}C-Phenylalanine radioactivity in collected plasma of individual piglets receiving different levels of tryptophan

4.4.4 Breakpoint Analysis

To determine tryptophan requirements, a two phase linear regression crossover model was used, in which data points were partitioned between two regression lines. The data partitioning selected was based on the model that produced the highest regression coefficients and the lowest residual error. The breakpoints and corresponding confidence intervals for enterally fed piglets receiving IG tracer are summarized in Table 4.5. The breakpoint estimated by breath $^{14}CO_2$ was determined to be 0.111 g tryptophan/kg/d (95% CI: 0.068-0.154), by percentage of dose oxidized was 0.113 g tryptophan/kg/d (95% CI: 0.072-0.154), and by plasma phenylalanine oxidation was 0.117 g tryptophan/kg/d (CI: 0.071-0.163). All of these measures yielded similar requirement estimates. Phenylalanine balance data did not exhibit a suitable pattern for breakpoint analysis, and so was not included in this analysis.

The breakpoints and respective confidence intervals for enterally fed piglets given IV tracer have been discussed previously in section 3.4.4, and are shown in Table 4.6. The breakpoint estimated by breath $^{14}CO_2$ was 0.122 g tryptophan/kg/d (CI: 0.085-0.159), by percentage of dose oxidized was 0.127 g/kg/d (CI: 0.089-0.164), by phenylalanine oxidation was 0.101 g/kg/d (CI: 0.081-0.121) and by phenylalanine balance was 0.103 g/kg/d (CI: 0.083-0.124). All of these measures yielded similar estimates due to the fact that the standard error of the estimate (SEE) for each of these measures were seen to overlap.

Table 4.5 Tryptophan Requirement by Breakpoint Analysis in Enterally Fed Piglets (IG Tracer)

Parameter	Breakpoint (g/kg/d)	CI_{lower} (g/kg/d)	CI_{upper} (g/kg/d)	r^2	Root MSE	SE of Estimate
$^{14}CO_2$	0.111	0.068	0.154	0.53	128.4×10^3	0.025
Phenylalanine Oxidation	0.117	0.071	0.163	0.57	6.04	0.027
% Dose Oxidized	0.113	0.072	0.154	0.56	1.86	0.024

Table 4.6 Tryptophan Requirement by Breakpoint Analysis in Enterally Fed Piglets (IV Tracer)

Parameter	Breakpoint (g/kg/d)	CI_{lower} (g/kg/d)	CI_{upper} (g/kg/d)	r^2	Root MSE	SE of Estimate
$^{14}CO_2$	0.125	0.098	0.152	0.68	121.7×10^3	0.016
Phenylalanine Oxidation	0.113	0.085	0.141	0.72	4.15	0.016
Phenylalanine Balance	0.114	0.077	0.150	0.72	5.42	0.073
% Dose Oxidized	0.127	0.089	0.164	0.71	1.66	0.022

4.5 DISCUSSION

Plasma concentrations of amino acids appeared to be similar for enterally fed piglets receiving either IG or IV tracer. As seen in the previous study, however, plasma phenylalanine, tyrosine, glutamine and taurine concentrations were significantly greater at tryptophan intakes below 0.10 g/kg/d compared to concentrations of these amino acids when tryptophan was provided at or above the estimated requirement (Tables 4.1, 4.2). This observation suggests that when tryptophan is supplied at the lowest treatment levels, protein synthesis is dramatically limited, resulting in saturation of amino acid catabolic pathways, and subsequent accumulation of phenylalanine and tyrosine in plasma. The rise in observed glutamine concentrations in plasma in the lowest tryptophan diet groups is likely due to its role in the transfer of nitrogen in the body. Finally, it is unclear as to why taurine concentrations were affected by dietary tryptophan intakes.

Estimates of phenylalanine kinetics were affected by the route of isotope infusion (Table 4.7). Although phenylalanine intake was not significantly different between enterally fed piglets receiving either IG or IV tracer, phenylalanine SRA (or plasma enrichment of labelled phenylalanine) was significantly higher for piglets given enteral diet and IV tracer ($p<0.05$) (Figure 4.8). Previous experiments in adults have also reported higher plasma enrichments in both fasted and fed subjects receiving IV compared to IG tracers (Hoerr et al., 1991; Matthews et al., 1993, 1999). Splanchnic utilization of phenylalanine is likely the reason for this observed difference. When labelled phenylalanine was given IG, a substantial proportion may have been taken up by the gut

Table 4.7 Estimates of Phenylalanine Kinetics: Comparison of Enterally Fed Piglets (IG Tracer) with Enterally Fed Piglets (IV Tracer) ϕ

Parameter	p Value*	Oral Diet / IG Tracer Means	Oral Diet / IG Tracer SEE	Oral Diet / IV Tracer Means	Oral Diet / IV Tracer SEE
Corrected $V^{14}CO_2$ ($x10^3$ DPM/kg/h)	NS	58.9	10.2	36.5	4.8
Plasma Phe SRA ($x10^3$ DPM/μmol)	0.023	39.0	1.7	46.9	2.9
Plasma Tyr SRA ($x10^3$ DPM/μmol)	NS	2.6	0.7	2.4	0.3
Tyr:Phe	NS	0.1	0.0	0.1	0.0
Phe Flux (Q) (μmol/kg/h)	0.005	199.2	14.3	145.8	12.0
Phe Oxidation (E) (μmol/kg/h)	0.037	1.5	0.2	0.9	0.2
Phe Intake (I) (μmol/kg/h)	NS	102.4	0.9	103.4	1.4
Phe Balance (I-E) (μmol/kg/h)	NS	101.0	0.9	102.6	1.3
% Dose Oxidized	NS	0.8	0.2	0.6	0.1

* NS = not significant, $p > 0.05$;

ϕ Means and SEE calculated for piglets receiving adequate tryptophan intakes (diets containing 0.15- 0.40 g Trp/kg/d)

on first pass, resulting in less label appearing in plasma. Indeed, Stoll et al. (1998) determined that ~35% of labelled phenylalanine was taken up by the splanchnic bed following the enteral infusion of diet with ^{13}C-algal protein. Another kinetic parameter, phenylalanine flux, was also affected by gut utilization of IG tracer. Flux was calculated as the amount of label infused divided by plasma phenylalanine SRA. As expected, piglets receiving enteral diet and IG tracer had significantly higher plasma phenylalanine fluxes compared to enterally fed piglets given IV tracers ($p<0.05$) (Figure 4.9). This result is similar to observations in fasted and fed adults given IG or IV tracers (Krempf et al., 1990; Hoerr et al., 1991; Sanchez et al., 1995; 1996; El-Khoury et al., 1998).

The subsequent estimate of phenylalanine oxidation calculated from plasma SRA was affected by the differences in plasma SRA between the piglets given IG or IV tracer ($p<0.05$). Calculated phenylalanine oxidation was determined as the rate of $^{14}CO_2$ production divided by plasma phenylalanine SRA. Previous oxidation studies, using either ^{13}C-lysine or ^{13}C-leucine tracers, did not demonstrate differences in labelled amino acid oxidation. El-Khoury et al (1998) compared lysine oxidation rates in adult men receiving ^{13}C-lysine either IG or IV. They found significantly higher plasma lysine fluxes in those given IG tracer, but no differences in plasma lysine oxidation calculated from plasma enrichment. When ^{13}C-leucine was administered to adults (Hoerr et al., 1991; 1993) or dogs (Yu et al., 1990; 1992), calculated leucine oxidation was not significantly different between those subjects or animals receiving the tracer either IV or IG.

Bross et al (1998) compared phenylalanine kinetics from several independent

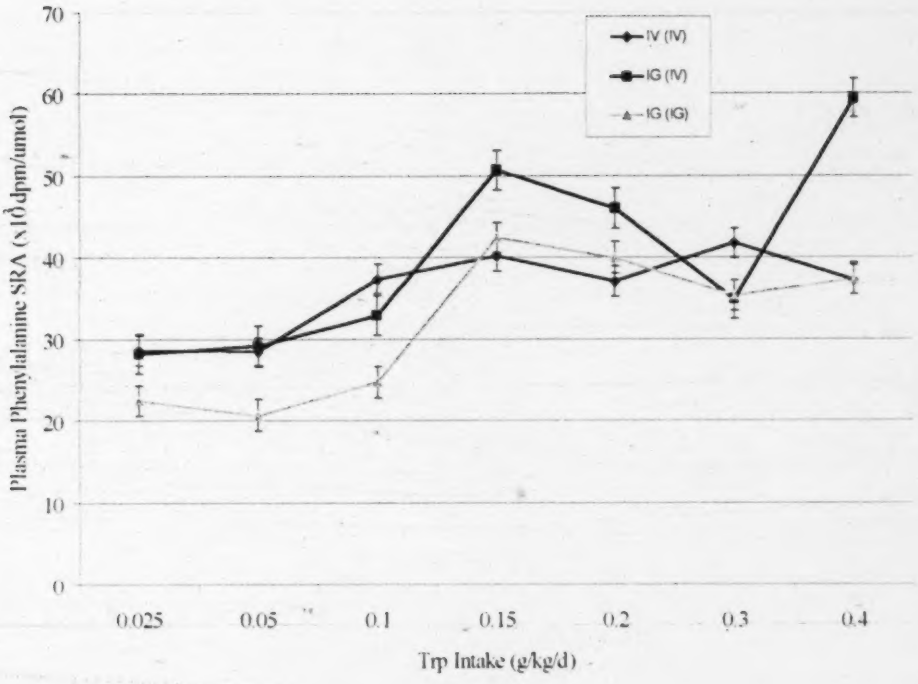

Figure 4.8 Plasma Phenylalanine Specific Radioactivity (SRA) of Piglets Receiving Parenteral Diet and Tracer (IV (IV)), Enteral Diet and IV Tracer (IG (IV)), or Enteral Diet and Tracer (IG (IG)). Data expressed as means ± pooled SE.

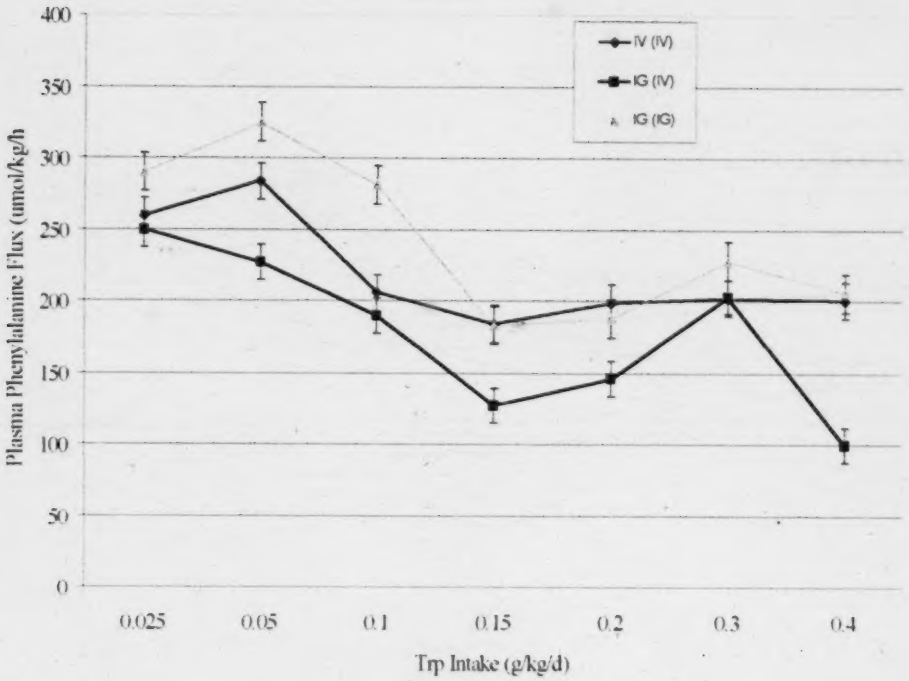

Figure 4.9 Plasma Phenylalanine Flux of Piglets Receiving Parenteral Diet and Tracer (IV (IV)), Enteral Diet and IV Tracer (IG (IV)), or Enteral Diet and Tracer (IG (IG)). Data expressed as means ± pooled SE.

studies in which adults were given ^{13}C-phenylalanine either IV or IG, in combination with enteral feedings. Calculated phenylalanine oxidation rates were similar between all studies, in which phenylalanine intake was constant in relation to total protein intake, regardless of the route of isotope infusion (Bross et al., 1998).

Others, however, have reported significant differences in phenylalanine oxidation in adults receiving IG vs IV infusion of ^{13}C-phenylalanine (Sanchez et al., 1995; 1996). Sanchez et al (1995) calculated phenylalanine oxidation rates to be 3.32 ± 0.76 and 1.16 ± 0.32 μmol/kg/h for enterally fed adults receiving ^{13}C-phenylalanine IG or IV, respectively. Thus, the IG infused tracer resulted in a phenylalanine oxidation estimate which was approximately 64 % greater than that of the IV tracer, and was reported to be statistically different (Sanchez et al., 1995). Our mean calculated phenylalanine oxidation rates for piglets receiving adequate dietary tryptophan (0.15-0.40 g/kg/d) was found to be 1.48 ± 0.89 and 0.85 ± 0.57 μmol/kg/h for animals given IG and IV tracers, respectively. We found that piglets infused IG with the phenylalanine tracer had ~ 45 % higher calculated phenylalanine oxidation rate than piglets receiving IV tracer. These differences in calculated phenylalanine oxidation rates are likely due to phenylalanine flux rates. As mentioned previously, phenylalanine oxidation was calculated as the rate of breath $^{14}CO_2$ production divided by plasma SRA. Alternatively, this calculation can be expressed as:

Phe Oxidation = (corrected V$^{14}CO_2$/kg/h) / dose (dpm/kg/h) * Flux

Due to the fact that the only difference in treatments between piglets was that of isotope infusion route, it is clear that actual phenylalanine oxidation rates do not differ between

groups given IG or IV tracers, only the calculated estimate of phenylalanine oxidation changed. When phenylalanine oxidation is expressed as a proportion of flux (which is analogous to % dose oxidized), oxidation was seen to account for 0.8 ± 0.57 and 0.6 % ± 0.28 of IG and IV infused tracer ($p>0.05$). Indeed, Sanchez et al (1995) reported the ratio of phenylalanine oxidation to phenylalanine flux for subjects given IG and IV tracers to be 0.04 ± 0.01 (or 4%) and 0.06 ± 0.03 (or 6%), respectively. Clearly, although this study and Sanchez et al (1995) found significant differences in plasma phenylalanine oxidation rates when IG and IV tracers were used, there were no significant differences in tracer oxidation when expressed as a % of dose oxidized.

Changes in plasma enrichment (SRA) and flux between IG and IV tracer in adults and piglets identically treated suggest that plasma is not an appropriate precursor pool from which to sample during amino acid oxidation studies. Phenylalanine oxidation, when expressed as % of dose, is likely the most accurate measure. This is due to the fact that both the dose of tracer infused and the subsequent collection of label in breath can be confidently determined and involve low levels of experimental error. As mentioned previously, % dose oxidized was not significantly different between enterally fed piglets given IG or IV tracers. In addition, the rate of $^{14}CO_2$ production was similar between the two alternative tracer groups ($p>0.05$). Therefore, ^{14}C-phenylalanine oxidation rates determined by the appearance of $^{14}CO_2$ in breath provides more accurate estimates of whole body phenylalanine oxidation.

Ultimately, the most important question to be addressed is whether the route of

tracer administration significantly affects the estimate of tryptophan requirement. Tryptophan requirements, expressed as plasma phenylalanine oxidation, $^{14}CO_2$ production rates, or percentage of dose oxidized, were similar for piglets infused with isotopically labelled phenylalanine IG or IV during enteral feeding (Tables 4.5, 4.6). The mean tryptophan requirement for enterally fed piglets determined by a 2-phase linear regression crossover model and based on % dose oxidized was found to be 0.113 ± 0.024 and 0.127 ± 0.022 g/kg/d for animals given IG and IV tracers, respectively. Therefore, although the route of tracer administration alters estimates of phenylalanine SRA and flux, ultimately the tryptophan requirement, as determined by the IAAO technique, remains unaffected.

5.0 GENERAL SUMMARY AND FUTURE DIRECTIONS

Low birth weight infants require unique nutritional management early in life due to the metabolic immaturity of the gastrointestinal tract and biochemical pathways. The use of the piglet model is a crucial step in determining amino acid requirements for both the parenterally and enterally fed premature infant. Although the requirement for other amino acids, such as threonine and methionine, are substantially greater (approximately 2 fold) in enteral versus TPN fed piglets, this appears not to be true for tryptophan. The mean tryptophan requirement was determined to be 0.127 ± 0.022 and 0.145 ± 0.023 g/kg/d (based on % dose oxidized) for enterally and parenterally fed piglets, a difference of about 12%. This is further evidence that all indispensable amino acids are not equally utilized by the gut on first pass, and thus the enteral and parenteral requirement for each indispensable amino acid must be empirically determined in the neonatal piglet.

Commercial amino acid solutions currently used to make TPN contain a large range of tryptophan concentrations. When adjusted to the amino acid intake of the piglets in this study, the solutions provide 0.20-0.32 g tryptophan/kg/d. This study suggests that these solutions provide 160-250% of the requirement. However, before it can be recommended that tryptophan levels be lowered in TPN formulas, neonatal needs for branched chain amino acids (isoleucine, leucine and valine) must be defined. Tryptophan competes with both branched chain and aromatic (phenylalanine and tyrosine) amino acids for transport in the gut and brain. Due to the fact that uptake of these amino acids into

brain depend on their relative concentration in plasma, the appropriate ratio of tryptophan to branched chain and aromatic amino acids must be determined. Until this is done, lowering tryptophan concentrations in TPN solutions cannot be recommended.

Ultimately, the goal is to determine the ideal amino acid profile for the low birth weight infant, so that protein synthesis is maximized and metabolic imbalances are reduced to a minimum. Once all of the indispensable amino acids are defined in the neonatal piglet and the ideal profile is ascertained, the next step will be to determine if this profile achieved all of the needs of the low birth weight infant. Oxidation studies are not commonly conducted using premature infants due to the invasive procedures involved (ie: IV tracer infusion, frequent blood sampling, and prolonged adaptation to diets containing excess or deficient amounts of amino acids). Bross et al (1998) has argued that several of these difficulties can be overcome, making oxidation studies more feasible. They demonstrated that oxidation studies using oral ^{13}C-lysine or ^{13}C-phenylalanine, and measurement of breath and urine instead of blood could be used to estimate tracer kinetics, within an 8 hour period. In addition, no prior adaptation to an experimental amino acid diet was required (Bross et al., 1998).

The issue of whether orally administered isotopes affect tracer kinetics and subsequent requirement estimates was addressed in the current study. As expected, oral compared to IV infused ^{14}C-phenylalanine resulted in lower plasma phenylalanine SRA and higher plasma fluxes, likely due to first pass utilization of phenylalanine by the gut. The route of isotope infusion significantly alters estimates of phenylalanine kinetics in

identically treated animals further suggesting that plasma should not be considered an appropriate precursor pool. Phenylalanine oxidation rates based on breath $^{14}CO_2$ collection, and ultimately tryptophan requirement estimates, were not altered by the route of infusion of tracer. This study has provided further evidence that the IAAO technique is an excellent method for the determination of amino acid requirements. Along with the work of Bross et al.(1998), it is likely that this technique may be applied to the low birth weight infant once the ideal amino acid profile has been established in the neonatal piglet.

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7.0 APPENDIX:

Two Phase Linear Regression Crossover Model

The mean requirement for tryptophan was estimated by breakpoint analysis using a two phase linear regression crossover model, using the program of J.D. House (Ph.D. thesis, 1995). With this model, the data points are partitioned between two separate linear regression lines and the intersection of the two lines, or the breakpoint, is determined.

The SAS program used is shown below. Phenylalanine oxidation rates (pheox) were used as the dependent variable, and dietary tryptophan intake (trpin) was used as the independent variable. The values included on the first line were the rates of oxidation associated with tryptophan intakes of less than 0.05 g/kg/d, whereas the remaining data points were used for the second regression line.

Program Statement:

```
options ls=79 nodate pageno=1;
title1 'Tryptophan Requirement- Indicator';
title2 'for Diet B (0.05 g/kg/d)';
data two;
input pig trpin pheox;
dvalue=0;
if trpin < 0.05 then dvalue=1;
trpdval=trpin*dvalue;
cards;
```

** data entered as piglet number, tryptophan intake, and the oxidative response (% dose oxidized) **

```
;
proc print;
proc glm;
model pheox=trpin dvalue trpin*dvalue;
proc reg outest=out123 covout outsscp=outsums;
model pheox=trpin dvalue trpdval;
proc print data= out123;
proc print data= outsums;
```

Output Statement:

General Linear Models Procedure

Dependent Variable: PHEOX

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	28.63050177	9.54350059	13.50	0.0001
Error	22	15.55354438	0.70697929		
Corrected Total	25	44.18404615			

R-Square	C.V.	Root MSE	PHEOX Mean
0.647983	55.14969	0.8408206	1.5246154

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRPIN	1	16.25759919	16.25759919	23.00	0.0001
DVALUE	1	12.19771340	12.19771340	17.25	0.0004
TRPIN*DVALUE	1	0.17518919	0.17518919	0.25	0.6236

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRPIN	1	0.63544210	0.63544210	0.90	0.3534
DVALUE	1	3.58178659	3.58178659	5.07	0.0347
TRPIN*DVALUE	1	0.17518919	0.17518919	0.25	0.6236

Parameter	Estimate	T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT	1.33373569	3.04	0.0060	0.43818040
TRPIN	-1.92527291	-0.95	0.3534	2.03075557
DVALUE	2.65843268	2.25	0.0347	1.18107958
TRPIN*DVALUE	-16.40635975	-0.50	0.6236	32.95808135

Model: MODEL1
Dependent Variable: PHEOX

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	3	28.63050	9.54350	13.499	0.0001
Error	22	15.55354	0.70698		
C Total	25	44.18405			

Root MSE	0.84082	R-square	0.6480
Dep Mean	1.52462	Adj R-sq	0.6000
C.V.	55.14969		

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|
INTERCEP	1	1.333736	0.43818040	3.044	0.0060
TRPIN	1	-1.925273	2.03075557	-0.948	0.3534
DVALUE	1	2.658433	1.18107958	2.251	0.0347
TRPDVAL	1	-16.406360	32.95808135	-0.498	0.6236

OBS	_MODEL_	_TYPE_	_DEPVAR_	_RMSE_	INTERCEP	TRPIN	DVALUE	TRPDVAL	PHEOX
1	MODEL1	PARMS	PHEOX	0.8408	1.3337	-1.9252	2.658	-16.41	-1
2	MODEL1	COV	INTERCEP	0.8408	0.1920	-0.8037	-0.192	0.80	.
3	MODEL1	COV	TRPIN	0.8408	-0.8037	4.1239	0.803	-4.12	.
4	MODEL1	COV	DVALUE	0.8408	-0.1920	0.8037	1.394	-35.07	.
5	MODEL1	COV	TRPDVAL	0.8408	0.8037	-4.1239	-35.070	1086.24	.

OBS	_TYPE_	_NAME_	INTERCEP	TRPIN	DVALUE	TRPDVAL	PHEOX
1	SSCP	INTERCEP	28.000	4.1490	8.000	0.2510	60.820
2	SSCP	TRPIN	4.149	0.9399	0.251	0.0088	4.757
3	SSCP	DVALUE	8.000	0.2510	8.000	0.2510	41.650
4	SSCP	TRPDVAL	0.251	0.0088	0.251	0.0088	1.351
5	SSCP	PHEOX	60.820	4.7569	41.650	1.3507	350.171
6	N		28.000	28.0000	28.000	28.0000	28.000

Linear Regression Equation:

The equation used for the linear regression model was as follows:

$$Y = A1 + B1x + (A2-A1)D + (B2-B1)(Dx) + E$$

Where Y represents the individual observations for the dependent variable (amino acid oxidation), A1 and A2 represent the intercepts of the first and second lines, respectively, and B1 and B2 represent the slopes of the first and second lines, respectively. The first line, the line with slope, was given a D value of 1, and the second line, with minimal slope was given a D value of 0. E represents the residual error of the model.

The equations of the two lines were:

Line 1: $Y = (A1 + A2 - A1) + (B1 + B2 - B1)x$

Line 2: $Y = A1 + B1x$

Equating the two functions and solving for x or the crossover point yields:

$$X = -(A2-A1)/(B2-B1)$$

The parameters in the Output statement allow for the rapid determination of the breakpoint or crossover point as follows:

A1 = INTERCEP = 1.33736

B = TRPIN = - 1.925273

(A2-A1) = DVALUE = 2.658433

(B2-B1) = TRPDVAL = -16.406360

Therefore, the crossover point was calculated as:

Crossover or Breakpoint = -DVALUE/TRPDVAL = -2.658433/-16.406360

= 0.162 g/kg/d

95% Confidence Interval Program:

The safe level of intake of an amino acid was estimated to be one that would meet the needs of the upper 95% of the population. Therefore 95% confidence limits corresponding to the rate of amino acid oxidation, as affected by the amino acid intake, were determined using Fieller's theorem (as done by J.D. House, Ph.D. thesis, 1995).

SAS Program:

This program used the parameters from the Output statement corresponding to the crossover or breakpoint analysis.

BETA2 = DVALUE = 2.658

BETA12 = TRPDVAL = -16.406

VBETA2 = DVALUE * DVALUE = 1.395

VBETA12 = TRPDVAL * TRPDVAL = 1086.240

COVBETA = DVALUE * TRPDVAL = -35.071

Program Statement:

```
options ls=79 nodate pageno=1 formdlim='_';
title1 'Tryptophan Requirement During TPN';
title2 '95% Confidence Interval- Oxidation Rate';
data one;

beta2= 2.658;
beta12= -16.406;
vbeta2= 1.395;
vbeta12= 1086.240;
covbeta= -35.071;
t=1.725;
ratio=-(beta2/beta12);
aa=vbeta2/(beta2**2);
bb=vbeta12/(beta12**2);
ab=covbeta/(beta2*beta12);
varratio=(ratio**2)*(aa+bb-2*ab);
seratio=sqrt(varratio);
clower=ratio- t*seratio;
cupper=ratio+ t*seratio;
proc print;
run;
```

Output Statement:

Tryptophan Requirement During TPN

95% Confidence Interval- Oxidation Rate

OBS	BETA2	BETA12	VBETA2	VBETA12	COVBETA	T	RATIO	AA
1	2.658	-16.406	1.395	1086.24	-35.071	1.725	0.162	0.197

OBS	BB	AB	VARRATIO	SERATIO	CLOWER	CUPPER
1	4.03571	0.80425	0.068894	0.26248	-0.29076	0.61478

Where RATIO = Breakpoint or crossover estimate

CLOWER = Lower 95% confidence value

CUPPER = Upper 95% confidence value = safe level of intake

= 0.615 g/kg/d